STUDIES ON ANTIDIABETIC AND OTHER BIOLOGICAL ACTIVITIES OF *CENTRATHERUM ANTHELMINTICUM*

ADITYA ARYA

FACULTY OF MEDICINE

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ADITYA ARYA

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Abstract

In search of phytomedicine for diabetes, this preliminary screening was conducted on twelve (12) different plants for hypoglycemic activity and antioxidant potential in the *in vitro* and *in vivo* study models. Amongst all, *Centratherum anthelminticum* seeds demonstrated maximum hypoglycemic and antioxidant potential. Therefore, the defatted crude methanolic fraction of the *Centratherum anthelminticum* seeds (CAMFs) was selected for detailed antidiabetic activity on the *in vitro* cell-based biological studies on mouse pancreatic cells, β-TC6 and on type 1 and type 2 diabetic rats for 4-weeks. The results of this study indicate that CAMFs showed potential antidiabetic effect on β-TC6 cells and confirmed beneficial effects on type 2 diabetic rats by ameliorating hyperglycemia and augmenting insulin secretion without any toxic signs and lethality in acute toxicity studies.

Furthermore, CAMFs was studied for H_2O_2-induced nuclear factor-κB (NF-κB) translocation effects on β-TC6 cells to investigate ROS-associated oxidative stress. Subsequently, a 12-week study was conducted on type 2 diabetic rats to determine biochemical parameters and their associated complications. The results of this study showed that CAMFs prevented H_2O_2-induced NF-κB translocation in the β-TC6 cells and downregulated hyperglycemia, oxidative stress, dyslipidemia and inflammatory response in diabetic rats.

Next, the crude fractions of *Centratherum anthelminticum* seeds were evaluated for pleotropic biological activities which had not been investigated on this plant. The result of this study showed that, defatted crude chloroform fraction (CACF) posesses the highest level of pleotropic bioactivities. CACF was tested on RAW264.7 macrophages for tumor necrosis factor-α (TNF-α) inhibition and for cytotoxic effects on targeted A549, PC-3, MCF-7, and WRL-68 cancer cell lines, along with antioxidant

potential on DPPH, FRAP and ORAC assays. Results of this study demonstrated that CACF exhibited the strongest inhibitory effects on TNF-α secretion and possessed maximum cytotoxic effects on MCF-7 cancer cells, with antioxidant abilities. Furthermore, we observed dose-dependent inhibitory response of CACF on serum TNF-α inhibition on lipopolysaccharides (LPS)-stimulated SD rats. Moreover, in line with the cytotoxic effects, CACF showed inhibitory role against the activation of NF-κB translocation in MCF-7 cells, dose-dependently.

It was concluded that CAMFs may be of interest in the pharmaceutical industry to be developed as a dietary supplement for the management of type 2 diabetes and its associated complications. Moreover, CACF fraction could be suggested for the alleviation of elevated inflammatory response as well as in the management of breast cancer and oxidative stress.

Abstrak

Dalam mencari ‘phytomedicine’ untuk diabetes, kami menjalankan penyaringan awal pada 12 tumbuhan yang berbeza untuk aktiviti hipoglisemik dan potensi antioksidan dalam model kajian *in vitro* dan *in vivo*. Di antara semua, biji-biji *Centratherum anthelminticum* menunjukkan potensi maksimum hipoglisemik dan antioksidan. Oleh itu, ekstrak metanol mentah biji *Centratherum anthelminticum* yg dihilangkan lemak (CAMFs) telah dipilih untuk kajian terperinci berasaskan sel biologi ke atas aktiviti antidiabetis secara *in vitro* pada sel pankreas tikus, β-TC6 dan tikus diabetis Jenis 1 dan Jenis 2, selama 4 minggu. Hasil kajian ini menunjukkan bahawa CAMFs mempamerkan keupayaan kesan antidiabetis ke atas sel-sel β-TC6 dan disahkan kesan yang baik pada tikus diabetik Jenis 2 boleh memperbaiki hiperglisemia dan menambahkan rembesan insulin tanpa sebarang tanda-tanda toksik dan maut dalam kajian toksisiti akut.

Tambahan pula, CAMFs dikaji untuk faktor nuklear -κB diaruh oleh H_2O_2 (NF-κB) kesan translokasi atas sel-sel β-TC6 untuk menyiasat tekanan oksidatif berkaitan ROS. Selepas itu, satu kajian 12-minggu telah dijalankan ke atas tikus diabetis jenis 2 untuk menentukan parameter-parameter biokimia dan komplikasi yang berkaitan. Keputusan kajian ini menunjukkan bahawa CAMFs menghalang translokasi faktor nuklear NF-κB diaruh oleh H_2O_2 dalam sel-sel β-TC6 dan menurunkan hiperglisemia, tekanan oksidatif, dislipidemia dan tindak balas keradangan pada tikus diabetis.

Seterusnya kami menilai biji *Centratherum anthelminticum* untuk aktiviti biologikal pleotropik yang tidak pernah disiasat pada tumbuhan ini. Hasil kajian kami menunjukkan bahawa ekstrak klorofom mentah yang dihilangkan lemak (CACF) mempunyai bioaktiviti pleotropik pada tahap tertinggi. CACF telah diuji pada makrofaj RAW264.7 untuk perencatan tumor nekrosis faktor α (TNF-α) dan untuk kesan

sitotoksik disasarkan pada barisan sel kanser A549, PC-3, MCF-7, dan WRL-68 , bersama-sama dengan potensi antioksidan pada cerakinan DPPH , FRAP dan ORAC. Keputusan kajian ini menunjukkan bahawa CACF mempamerkan kesan rencatan tertinggi pada rembesan TNF-α dan mempunyai kesan sitotoksik maksimum pada sel-sel kanser MCF-7, dengan keupayaan antioksidatif. Tambahan pula, kami perhatikan respon perencatan bergantung kepada dos pada CACF ke atas perencatan serum TNF-α pada lipopolysaccharides (LPS) dirangsang SD tikus. Selain itu, selaras dengan kesan sitotoksik, CACF menunjukkan peranan perencatan terhadap pengaktifan translokasi NF-κB dalam MCF-7 sel, bergantung pada dos.

Sebagai rumusan, kami membuat kesimpulan bahawa penggunaan CAMFs mungkin penting dalam industri farmaseutikal untuk dibangunkan sebagai diet tambahan bagi pengurusan diabetes jenis 2 dan komplikasi yang berkaitan dengannya. Selain itu, ekstrak CACF boleh dicadangkan untuk mengurangkan tindak balas keradangan yang tinggi serta dalam pengurusan kanser payudara dan stress oksidatif.

Synopsis

In search of phytomedicine for diabetes, the leaf methanolic extract of nine (9) different plants were screened for the hypoglycemic activity, such as *Centratherum anthelminticum*, *Cissus quadrangularis*, *Madhuca indica*, *Parthenium hysterophorus* L., *Sida acuta* Burm F., *Vicoa indica* Cass, *Vanda tessellata* (Roxb.), *Woodfordia fruticosa* Kurz and *Xanthium strumarium* L. The screening consisted of *in vivo* acute toxicity studies and hypoglycemic screening on normoglycemic rats through the intraperitoneal (i.p) or oral routes of administration, following the intraperitoneal glucose tolerance test (IPGTT) and oral glucose tolerance test (OGTT). From these screening tests, it was observed that the leaf extracts of *Centratherum anthelminticum*, *Cissus quadrangularis* and *Woodfordia fruticosa* Kurz possess maximum hypoglycemic effect without any acute toxic signs or lethality.

Next, we studied three (3) plants, *Terminalia arjuna*, *Terminalia bellerica* and *Terminalia chebula* for their antioxidant properties in the *in vitro*, 1,1-diphenyl-2-picrylhydrazyl (DPPH), oxygen radical absorbance capacity (ORAC) and ferric reducing/antioxidant power (FRAP) assays, along with acute toxicity and oral hypoglycemic tests on normal rats. The results of these tests indicate that the methanolic leaf extracts of the selected plants possess antioxidant and hypoglycemic properties, without any toxic effects. This positive effect may be due to the poly-phenolic compounds shown to be present in *Terminalia* species.

Furthermore, by screening different parts of such selected plants, it was found that *Centratherum anthelminticum* seeds showed maximum hypoglycemic and antioxidant potential. Therefore, the seeds of this plant were used for detailed antidiabetic research including targeted biological studies.

The defatted crude methanolic fraction of the *Centratherum anthelminticum* seeds (CAMFs) was selected for detailed antidiabetic studies on *in vitro* cell-based mechanistic approaches, including short-term studies on diabetic animals. CAMFs, was initially tested on mouse pancreatic β-TC6 cell line for cellular viability and proliferation, with 2-NBDG glucose uptake, insulin secretion and glucose transporter (GLUT-1, 2 and 4) protein expression. Next, CAMFs was subjected to acute toxicity studies through intraperitoneal (i.p) and oral routes of administration, following a four-week study on Streptozotocin (STZ)-induced type 1 and STZ-nicotinamide-induced type 2 diabetic rats to determine blood glucose and insulin levels measuring the animals' food and water intake as well as body weight.

The results of this study on β-TC6 cell-line models demonstrate the non-cytotoxic nature of CAMFs on β-TC6 cell proliferation. Furthermore, it showed dose-dependent increase in glucose uptake, as well as insulin secretion was mediated by upregulating GLUT-2 and GLUT-4 protein expression. CAMFs significantly reduced hyperglycemia and augment insulin secretion in type 2 diabetic rats, without any toxicity in acute studies. In contrast, type 1 diabetic rats did not show much improvement in glycemic control with non-significant increase in insulin secretion. The antidiabetic effects of CAMFs may be attributed to its poly-phenolic constituents, as identified by the LCMS-MS as the major compounds.

In continuation with the present study, an attempt was made to gain a better understanding of the effects of CAMFs in ROS-associated oxidative stress on insulin resistance signaling pathways by investigating the role of CAMFs in H_2O_2-induced NF-κB activation on β-TC6 cells. The result of this study showed that CAMFs prevent H_2O_2-induced NF-κB translocation in the β-TC6 cells. Next, to confirm the beneficial effects of CAMFs produced by the β-TC6 cell line and animal model of type 2 diabetic rats, a 12-week (sub-chronic) study was carried out on type 2 diabetic complications in

STZ-nicotinamide-induced type 2 diabetic rats to determine biochemicals, enzyme and physiological parameters with markers of oxidative stress and pro-inflammatory cytokines.

Results of this study revealed that CAMFs significantly reduced hyperglycemia by augmenting serum insulin, C-peptide, total protein, and albumin levels. Moreover, elevated glycated hemoglobin, lipids and enzyme activities of serum and tissues were also restored to near normal. CAMFs produced antioxidant effect by elevating glutathione (GSH) and by down-regulating malondialdehyde (MDA) levels in the tested serum and tissues. CAMFs also caused dose-dependent reduction of elevated pro-inflammatory cytokines, tumor necrosis factor α (TNF-α) levels, as well as that of interleukin (IL)-1β and IL-6 in the serum and tissues of the diabetic rats. Histopathological studies showed that long term administration of CAMFs for 12 weeks prevent STZ-induced structural degeneration in the liver, kidney and pancreas of diabetic rats. Overall results established that CAMFs exert apparent antidiabetic effect suggesting that it may be a valuable candidate for insulin-resistant type 2 diabetes and its associated complications such as oxidative stress, dyslipidemia as well as in inflammatory response.

Apart from antidiabetic property, several studies have investigated different pharmacological effects of the seeds of this plant in different ailments but investigations on the inflammatory cytokines and cytotoxic effects on different cancer cell lines have not been reported in the literature. Therefore, this study was planned to investigate the biological activities related to the inflammatory response on cytokines and cancer cells.

The chloroform fraction of the *Centratherum anthelminticum* seeds (CACF) was investigated on RAW264.7 macrophage cells for the TNF-α inhibition response with cytotoxic effects on the targeted A549, PC-3, MCF-7, and WRL-68 cells. The study

results demonstrated that CACF inhibits TNF-α secretion in stimulated RAW264.7 macrophage cells without affecting their viability. Cytotoxicity screening results indicate that among all the cell lines tested, CACF displayed maximum inhibitory effect on the breast cancer (MCF-7) cells.

Furthermore, in line with the inhibitory effects of CACF on the TNF-α secretion, the effects on serum TNF-α in an *in vivo* model was confirmed; the results of the study demonstrate that CACF showed dose-dependent inhibitory effects on serum TNF-α in LPS-stimulated SD rats, without any toxic signs and lethality in acute toxicity studies. Next, the effectiveness of CACF on a targeted MCF-7 cell line was tested by evaluating the linkage between NF-κB activation on the MCF-7 cells through a mechanistic approach. The results of this study showed that CACF dose-dependently inhibits the activation of NF-κB translocation in MCF-7 cells. Alongwith all these tests, the antioxidant potential of the CACF on DPPH, ORAC and FRAP assays were evaluated. Results of these tests showed that CACF also possesses antioxidant abilities and the chemicals inside CACF could be responsible for such pleotropic biological activities.

This study suggests that *Centratherum anthelminticum* seeds are corroborated with potential biological activities; this was exhibited in the defatted crude methanolic fraction (CAMFs) which might serve as a promising alternative as nutraceutical (dietary supplement) in the management of type 2 diabetes and in its associated complications. Moreover, crude chloroform fraction (CACF) also showed its potential to be effective in the management of inflammation and breast cancer with oxidative stress conditions.

An overview of the research methods and approaches employed in this study is shown in Figure 3.1.

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On the personal front, I would like to sincerely thank my beloved parents; my father, Kamal Kishore, and my mother, Shivkanti. I am dedicating this dissertation in my mother's memory; she passed away a few years ago after being diagnosed with leukemia. My parents have always believed in me and have always pushed me to dream, but they more importantly are the proof that by working hard you can achieve your dreams. I would like to thank my sisters' Shraddha, Jyoti, Sheetal, Archana and brother Amit, for their support throughout this process. And last, but certainly not the least, i would like to thank my wife, Nitika and my loving sons' Amritam and Ashmit, for their love and affections throughout my graduate studies; My wife is the source of my drive to be a better person everyday and my sons' turned my life towards enthusiasm and energy.

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%	Percentage
i.e	For example
±	Plus/minus
α	Alpha
β	Beta
g	Gram
g/kg	Gram per kg
bw	Body weight
GAE/g	Gallic acid equivalent per gram
mg	Milligram
mg/kg	Milligram per kilogram
mg C/g	Milligram catechin per gram
mM	Millimolar
mmol/L	Millimolar per litre
nm	Nanometer
µg/mL	Microgram per millilitre
µM	Micromolar
Q/g	Quercetin per gram
TE/mL	Trolox equivalent per millilitre
γ-GT	γ-glutamyl transpeptidase
2-NBDG	2-[N-(7-nitrobenz-2-oxa-1,3-diaxol-4-yl)amino]-2-deoxyglucose
ALP	Alkaline phosphatase
ALT	Alanine transaminase
AST	Aspartate transaminase

CACF	Chloroform fraction of *Centratherum anthelminticum* seeds
CAMFs	Methanolic fraction of *Centratherum anthelminticum* seeds
CD14	Glycophosphotphatidylinositol-linked protein
CRP	C-reactive protein
DM	Diabetes mellitus
DM1	Type 1 diabetes mellitus
DM2	Type 2 diabetes mellitus
DNA	Deoxyribonucleic acid
DPPH	2, 2-diphenyl-1-picryl-hydrazyl
FFA	Free fatty acid
FRAP	Ferric reducing antioxidant power
GC-MS	Gas chromatography mass spectrometry
GLUT	Glucose transporter protein
GSSG	Oxidized glutathione
HbAlc	Glycated hemoglobin
HDL-C	High density lipoprotein cholesterol
H_2O_2	Hydrogen peroxide
IC_{50}	Inhibitory concentration upto 50%
IDDM	Insulin dependent diabetes mellitus
IL-1β	Interlukin-1β
IL-6	Interlukin-6
iNOS	Nitric oxide synthase
i.p	Intraperitoneal
IPGTT	Intraperitoneal glucose tolerance test
LBP	Lipopolysaccharide-binding protein
LCMS-MS	Liquid chromatography-tandem mass spectrometry

LDL-C	Low density lipoprotein-cholesterol
LPS	Lipopolysaccharide
MAPKs	Mitogen-activated protein kinase
MDA	Malondialdehyde
MTT	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NA	Nicotinamide
NAD	Nicotinamide adenine dinucleotide
NF-κB	Nuclear factor-κB
NIDDM	Non insulin dependent diabetes mellitus
NO	Nitric oxide
OGTT	Oral glucose tolerance test
ORAC	Oxygen radical absorbance capacity
ROS	Reactive oxygen species
RTCA	Real-Time Cellular Analysis
SD	Sprague dawley
STZ	Streptozotocin
TC	Total cholesterol
TFC	Total flavonoid content
TG	Triglycerides
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor- α
TPC	Total phenolic ontent
TPTZ	2,3,5-triphenyl-1,3,4- triaza-2-azoniacyclopenta-1,4-diene chloride
TTC	Total tannin content

CHAPTER 1

INTRODUCTION

1.1 Background

To date, a huge number of plants have been studied as potential source of phytomedicine in the management of diabetes, oxidative stress, inflammation, cancer with many other disorders. Plants which are utilized in our study for the management of diabetes mellitus (DM) are either used individually or in the form of mixture in many formulations in the Indian system of medicine including various herbal and traditional medicines.

The plants which were selected in this study are based on their traditional application within the region. We have selected the plants which are mostly utilized by the tribal community for the purpose of "Madhumeha" (Diabetes mellitus) with inflammatory conditions. These plants are mostly used by the Gond community near the satpuda ranges of forest in the surroundings areas such as Hoshangabad and Betul districts in the state of Madhya Pradesh, India, where these plants are grown in the forest or cultivated by the people for sale. The community use the leaf from these plants by boiling the fresh or dried leaves and consuming them orally by drinking the decoctions in the morning and evening.

The plants tested were *Centratherum anthelminticum*, *Parthenium hysterophorus* L., *Xanthium strumarium* L., and *Vicoa indica* Cass, which are all from the Asteraceae family, together with five others from different families: *Woodfordia fruticosa* Kurz (Lythraceae), *Sida acuta* Burm F. (Malvaceae), *Vanda tessellata* (Roxb.) (Orchidaceae), *Madhuca indica* (Sapotaceae), and *Cissus quadrangularis* (Vitaceae). Three plants were from the same genus *Terminalia* such as *T. arjuna*, *T. bellerica*, and

T. chebula from the Combretaceae family. The claim made by traditional healers made us include these plants in this study to scientifically validate such plants for their hypoglycemic potential, followed by selecting the most potent parts of the respective plants for other pleotropic biological evaluations as well. Some of these selected plants have already been scientifically validated and some of them have not. There are few reports on the hypoglycemic potential of different parts of *Centratherum anthelminticum* (Ani and Naidu., 2008; Fatima *et al*., 2010; Shah *et al*., 2008), *Parthenium hysterophorus* L. (Patel *et al*., 2008), *Cissus quadrangularis* (Chidambaram and Carani., 2010), *Woodfordia fruticosa* Kurz (Charve *et al*., 2010) and *Terminalia* species (Murali *et al*., 2007; Rao and Nammi., 2006; Sabu and Kuttan., 2002). Therefore, this study is focused on the scientific evaluations of the leaf extracts of the selected plants, followed by the selection of the most effective part of the single plant for the detailed pharmacological investigations.

1.2 Objectives of the study

The general research approach that was followed were; to initially screen 12 Indian plants used by tribal peoples as folkflore medicine for diabetes and for antioxidative abilities; eventually to focus on the most potent part of the single plant with the strongest hypoglycemic activity and to further assess the extract/fraction of the potential parts using the *in vitro* cell-based study alongwith *in vivo* animal models through preclinical approaches for the antidiabetic effects and its associated complications. In addition, further assessment of its other biological activities which have not yet been investigated on the same plants through *in vitro* and *in vivo* study models.

Therefore, the specific objectives of the study were:

a) To perform a hypoglycemic screening test on the selected plant extract/fraction using *in vivo* screening models on normal rats.

b) To select the most potent part with maximum hypoglycemic and antioxidant effects from the tested plants with positive results.

c) To test the selected part of the plant extract/fraction for antidiabetic effects on the *in vitro* cell-based mechanistic approaches in mouse pancreatic β-TC6 cells, alongwith short term study on the type 1 and type 2 diabetic animal models.

d) To examine the role of the selected extract/fraction in ameliorating complications associated with diabetes through long term administration on the *in vivo* animal model and to analyse the possible major compounds present in the extract/fraction.

e) To determine the potential biological activities which have not been investigated before in the selected part of the plant extract/fraction using cell-based *in vitro* studies alongwith *in vivo* model.

1.3 Findings from this research

1.3.1 Outcomes from the 1st published paper

In search of the most potent plant through the screening of twelve (12) different plants for hypoglycemic activity, we first investigated nine (9) such plants to evaluate the effects of the leaf extracts for antihyperglycemia. Initially, acute toxicity test and hypoglycemic screening were performed on normal rats whose blood glucose levels were measured previously following an oral or intraperitoneal (i.p.) administration of the extracts at different time periods. The results of acute oral and intraperitoneal

toxicity studies demonstrated that the methanolic extracts from all of the selected plant leaf except *Parthenium hysterophorus* L. were non-toxic up to a dose of 2500 mg/kg. Based on this finding, doses with 250 mg/kg and 500 mg/kg were chosen as the maximum dose for further experiments involving administration by the i.p and oral routes respectively. Among these, *Centratherum anthelminticum* (Asteraceae), *Cissus quadrangularis* (Vitaceae), and *Woodfordia fruticosa* Kurz (Lythraceae) significantly reduced postprandial blood glucose levels in normal glycemic rats ($P<0.001$), with slight reductions effected by *Sida acuta* Burm F. ($P = 0.002$) and *Parthenium hysterophorus* L. ($P = 0.017$). The extracts that reduced postprandial blood glucose levels both orally and i.p. in the hypoglycemic screening tests were evaluated further for glucose challenge through glucose tolerance tests with i.p and oral administration on overnight-fasted normal rats. The results of these tests showed that *Centratherum anthelminticum*, *Cissus quadrangularis*, and *Woodfordia fruticosa* extracts possess maximum hypoglycemic effects and could be suggested as a potential candidate for the management of postprandial hyperglycemia.

1.3.2 Outcomes from the 2nd published paper

Furthermore, three (3) different *Terminalia* species, *T. arjuna*, *T. bellerica*, and *T. chebula* leaves methanolic extracts were evaluated for their antioxidant and hypoglycemic potential. Initially, extracts were evaluated for their total phenolic, flavonoid, and tannin content, with *in vitro* antioxidant potential on DPPH, ORAC, and FRAP assays and hypoglycemic activities of the extracts were evaluated through hypoglycemic screening and oral glucose tolerance test (OGTT) on normal rats. Results of all these experiments revealed that the methanolic extract of *T. chebula* leaves contained the maximum amount of total phenolic content (TPC), followed by those of *T. bellerica* and *T. arjuna* (266.16, 259.28, and 147.23 mg GAE/g extract, respectively). Likewise, the total flavonoid content (TFC) were 29.23, 16.15, and 8.19 mg Q/g extract,

respectively. *T. chebula* possessed the highest content of tannin (TTC), followed by *T. arjuna* and *T. bellerica* (8.36, 4.68 and 6.31 mg C/g extract, respectively). The scavenging capacity of *T. chebula* for the antioxidant DPPH was the highest among the extracts tested, as it recorded the lowest IC_{50} value (11.6 μg/mL) value of all 3 extracts. The results demonstrated that *T. chebula* extract was found to possess highest oxygen radical absorption capacity with the lowest ORAC (18.23 μM TE/mL) value. In the FRAP assay, the ferric reducing antioxidant abilities of the extracts showed that *T. arjuna* > *T. chebula* > *T. bellerica*. This correlates the potential of polyphenolic content enriched with antioxidant capabilities and substantiates the results of the hypoglycemic screening and OGTT, which determined that the *T. chebula* extract had a maximum hypoglycemic effect on normal rats ($P<0.001$) compared to that of *T. bellerica* and *T. arjuna*, respectively. Altogether, the results of this study showed that selected *Terminalia* species possess hypoglycemic potential with antioxidative abilities. Thus, it may be concluded that the use of selected *Terminalia* species leaf as food supplements may help in reducing oxidative stress and elevated blood glucose levels.

1.3.3 Outcomes from the 3rd published paper

Based on the pilot screening result, the defatted crude methanolic fraction of the *Centratherum anthelminticum* seeds (CAMFs) was selected for further detailed studies on the mouse pancreatic β-TC6 cell line to determine its potential effect on diabetic conditions, thereby confirming its effect on type 1 and type 2 diabetic rat models.

Initially, CAMFs was tested on β-TC6 cells for cellular viability and proliferation, 2-NBDG glucose uptake and insulin secretion with glucose transporter (GLUT-1, 2 and 4) protein expression. The result of cellular viability assay showed that CAMFs concentrations of up to100 μg/mL did not exert cytotoxic effects on β-TC6 cell viability as compared to untreated control cells. Next, we monitored treated β-TC6 cells

for three days using Xcelligence Real-time Cell Proliferation (RTCA) assays. RTCA data showed that β-TC6 cells treated with 50, 25, 12.5, 6.25 or 3.125 µg/mL of CAMFs proliferated in a similar manner with control cells throughout the 72-h treatment.

The result of this study indicate that CAMFs increased glucose uptake by the endocytosis of fluorescent glucose analogue 2-NBDG in β-TC6 cells, and showed an increase in intracellular 2-NBDG fluorescence, which was visible in distinct groups of cells, suggesting that β-TC6 cells retain the heterogeneous glucose uptake activity of native β-cells. Interestingly, CAMFs increased insulin secretion in a dose-dependent manner at glucose concentrations of 6.25, 12.5, 25 and 50 mM. In particular, at 12.5 µg/mL CAMFs elicited a marked increase in insulin secretion in the β-TC6 cells. The expression of glucose transporter protein GLUT-1, GLUT-2 and GLUT-4 on β-TC6 cells were examined with western blotting analysis. The result demonstrated that CAMFs increased GLUT-2 and GLUT-4 protein levels in a dose-dependent manner compared to control β-actin, leading to increased glucose uptake in the pancreatic β-TC6 cells. In contrast, CAMFs did not alter GLUT-1 protein expression, suggesting the essential role of CAMFs in the translocation of insulin-regulated glucose into the cells.

The acute oral and i.p toxicity studies revealed the non-toxic nature of CAMFs; no lethality or toxic reactions were observed for any of the doses tested. Based on these findings, 100 mg/kg and 50 mg/kg doses were chosen as the maximum dose for further experiments involving i.p and oral routes of administration respectively on STZ-induced type 1 and STZ-nicotinamide-induced type 2 diabetic rats. The study results showed that CAMFs significantly reduced hyperglycemia in type 2 diabetic rats. At the end of the study week, 100 and 50 mg/kg doses of CAMFs produced the maximal decrease of blood glucose level (51.40% and 46.47%) respectively compared to the glibenclamide group, which achieved a 50% drop in blood glucose levels. At 100 and 50 mg/kg of CAMFs, type 1 diabetic rats demonstrated 23.21% and 21.05% reduction in blood

glucose levels at the end of the study week. In continuation with the insulin secretion in β-TC6, CAMFs significantly augmented insulin levels of type 2 diabetic rats, plausibly through enhancing glucose uptake and insulin secretion, whereas type 1 diabetic rats did not show significant improvement in the insulin secretion.

As mentioned above, CAMFs demonstrated antidiabetic potential on β-TC6 cells and type 2 diabetic rats plausibly through enhancing glucose uptake and insulin secretion. The antidiabetic potential may be attributed to CAMFs constituents as phytochemical studies by LCMS-MS identified certain poly-phenolic principles: quercetin glycoside, 3,4-0-dicaffeoylquinic acid, caffeic acid, naringenin-7-0-glucoside and kaempferol as the major compounds.

1.3.4 Outcomes from the 4th published paper

Moreover, to confirm the demonstrated activity on β-TC6 cells and type 2 diabetic rats, further study was aimed to ascertain the potential of CAMFs in the management of type 2 diabetes and its associated complications. CAMFs, was initially tested on ROS-induced oxidative stress associated with insulin resistance signaling pathway to determine the NF-κB translocation effects in β-TC6 cells. The result of this study showed that treatment with 25 μg/mL of CAMFs significantly inhibited H_2O_2-induced NF-κB translocation from the cytoplasm into the nucleus, suggesting the antioxidative role of CAMFs in oxidative stres conditions.

Next, a 12-week sub-chronic CAMFs study was carried out on STZ-nicotinamide-induced type 2 diabetic rats to evaluate glycemia, essential biochemical parameters, lipid levels, oxidative stress markers, and proinflammatory cytokines level. This study result demonstrated that CAMFs significantly reduced hyperglycemia. The percentages of the inhibition of blood glucose effected by 50, 25, and 10 mg/kg doses of

CAMFs were 75.86%, 69.82%, and 65.08%, respectively, as compared to that of glibenclamide (68.53%) at the end of the study week.

Biochemical parameters result displayed that CAMFs significantly elevated serum insulin, C-peptide, total protein, and albumin levels in diabetic rats and inhibited glycated hemoglobin (HbA1c) significantly in a dose-dependent manner, indicating significant improvement of glycemic control by CAMFs. Moreover, CAMFs significantly down-regulated serum TG, TC, LDL-C, and FFA levels in a dose-dependent manner, whereas HDL-C levels were significantly elevated. Similarly, CAMFs produced normalization of the enzyme, AST, ALT, ALP, and γ-GT activities in the serum, liver, and kidney of diabetic rats, dose-dependently.

CAMFs revealed antioxidant potential as GSH levels in the liver and pancreas of diabetic rats were significantly elevated, although not much changes were observed in the kidney GSH levels. In contrast, CAMFs recorded maximum reduction of MDA levels in the liver of diabetic rats at all the doses, whereas in the kidney and pancreas, MDA levels showed a dose-dependent reduction.

Next, CAMFs role was determined on pro-inflammatory cytokines. The study results showed that the elevation caused to TNF-α, IL-1β, and IL-6 in diabetic rats were significantly inhibited by CAMFs. TNF-α levels in the pancreas, kidney and serum of diabetic rats were significantly down-regulated. Likewise, CAMFs significantly reduced the IL-1β and IL-6 levels in the pancreas, with non-significant reduction observed in the serum and kidney tissues. Histopathological studies showed that long term administration of CAMFs for 12-weeks prevented STZ-induced structural degeneration in the liver, kidney and pancreas of diabetic rats. The liver of diabetic rats treated with CAMFs showed improvement in the hepatocytes with less cytoplasmic vacuolization, along with recovery in the inflammed mononuclear cells as compared to diabetic control

rats. However, a few hepatocytes with cytoplasmic vacuolization and pyknotic nuclei were still seen. Similarly, the kidneys of diabetic rats treated with CAMFs showed mild recovery in the inflammation and less infiltaration of cells were seen in the renal parenchyma compared to untreated diabetic rats with mild tubular epithelial atrophy, as well as improvement in the congestion of capillaries. Likewise, the pancreas demonstrated an increase in the mass of islet cells with a reduction of necrosis, vacuolations, hydropic cells and pyknotic nuclei in diabetic rats treated with CAMFs.

Altogether, the *in vitro* and *in vivo* study results showed that CAMFs exerted apparent antidiabetic effects on pancreatic β-TC6 cells and type 2 diabetic rats. Therefore, it is concluded that CAMFs is a valuable candidate for insulin-resistant type 2 diabetes and could be suggested in its associated complications such as dyslipidemia, oxidative stress, and inflammatory response.

1.3.5 Outcomes from the 5th published paper

In line with other biological activities, *Centratherum anthelminticum* seeds chloroform fraction (CACF) was investigated for the pleotropic bioactivities by assessing antioxidant potential, cytotoxic effect, and TNF-α inhibition activity with the NF-κB activation response. The total phenolic content and antioxidant property of CACF was evaluated with DPPH, ORAC, and FRAP assays. The total phenolic content of CACF was observed to be 37.16 ± 0.85 μg GAE/mg extract. CACF exhibited a dose-dependent inhibition of DPPH activity with an IC_{50} value of 22.56 ± 1.4 μg/mL. Maximal DPPH scavenging activity occurred at 41 ± 1.2 μg/mL of CACF with an inhibition of 89%. Likewise, CACF showed a FRAP value of 1048.3 μmol/L, while the positive control Ascorbic acid and BHT displayed a value of 6240 and 907.7 μmol/L, respectively. CACF had an ORAC value of 992.34 ± 45.12 μM trolox equivalent at 20

μg/mL. On the other hand, quercetin had an ORAC value of 1018.00 ± 34.82 μM of Trolox equivalent at 5 μg/mL.

CACF effectively and dose-dependently inhibited TNF-α release *in vitro* and *in vivo*. CACF inhibited TNF-α secretion in stimulated RAW 264.7 macrophages with an IC_{50} of 0.012 μg/mL and exhibited maximal TNF inhibition of 90% at 0.31 μg/mL. This significant inhibitory effect was observed at non-cytotoxic doses ranging from 0.031 to 0.002 μg/mL, without affecting their viability. The acute toxicity study revealed the non-toxic nature of CACF. CACF showed a dose-dependent inhibitory effect of serum TNF-α in LPS-stimulated rats; the observed effect in rats pretreated with 100 mg/kg was almost 61%, compared to that of dexamethasone which was 67%. The cytotoxicity of CACF was tested using the MTT assay; CACF effective inhibitory concentrations (IC_{50}) for lung cancer (A549), prostate cancer (PC-3), breast cancer (MCF-7), and normal hepatic cells (WRL- 68) were 31.42 ± 5.4, 22.61 ± 1.7, 8.1 ± 0.9, and 54.93 ± 8.3 μg/mL, respectively. Furthermore, we focussed on MCF-7 cells for cytotoxic activity towards a mechanistic approach. When MCF-7 cells were treated with different concentration of CACF (8, 4, and 2 μg/mL), exhibited dose-dependent inhibitory effects against the activation of NF-κB translocation. In parallel, the morphological changes of NF-κB translocation indicated by immunofluorescence staining also showed an inhibitory effect of CACF on TNF-α-induced NF-κB translocation in a dose-dependent manner. Altogether, the results of this study demonstrate the potential of CACF in the management of elevated inflammatory cytokines TNF-α, including breast cancer and associated oxidative stress conditions.

CHAPTER 2

LITERATURE REVIEW (CRITICAL REVIEW)

2.1 Diabetes and its complications

Diabetes mellitus ranks among the top causes of mortality throughout the world (Wild *et al.*, 2004). This syndrome is initially characterized by loss of glucose homeostasis as a result of defects in insulin secretion, insulin action or both, leading to impaired metabolism of glucose and other energy-yielding fuels such as lipids and proteins (American Diabetes Association (ADA), 2011).

Diabetes is commonly referred to as type 1 (DM1) and type 2 (DM2). Both forms involve pancreatic islet β-cell abnormalities, characterized by death in type 1 and accelerated apoptosis in type 2. The global prevalence of diabetes was estimated to be 6.4%, affecting 285 million adults in the year 2010 and its projections estimated to be 7.7%, affecting 439 million adults in 2030 (Tamrakara *et al.*, 2011).

Malaysia is one of the top 10 countries in the estimation of diabetes prevalence in 2010 and 2030 at 11.6% and 13.8%, respectively (Shaw *et al.*, 2010). According to the National Health and Morbidity Survey III which was conducted in the year 2006, 15.5% of the general population in Malaysia was estimated to be suffering from chronic illnesses, where diabetes mellitus was recorded as the second most common chronic illness, constituting 4.0% out of 15.5% of the general population (Amal *et al.*, 2011).

As a chronic metabolic disorder, diabetes mellitus can affect all major organ systems of the body, leading to complications that may result in significant morbidity and premature mortality. Clinical, preclinical, and epidemiological studies indicate an association between oxidative stress and inflammation in the development of DM2 and its complications (Donath and Shoelson, 2011).

2.1.1 Oxidative stress

Environmental factors and chemicals play a major role in the etiology of diabetic complications. Oxidative stress is caused by an imbalance between the production of ROS and antioxidant defences within the ability of a biological system to readily detoxify the reactive intermediates or repair the resulting damage. Disturbances in this normal redox state can cause toxic effects through the production of peroxides and free radicals and simultaneous decline in antioxidant defence mechanisms which can cause damage to all components of the cell, including proteins, lipids, and DNA which may inhibit their normal function (Karaca *et al*., 2006).

Oxidative stress, as a consequence of hyperglycemia, changes in energy metabolism and inflammatory mediators play an important role in the pathophysiology of diabetes and its complications (Ferdinando and Michael, 2010). In DM2, production of ROS is increased due to insulin resistance and hyperglycemia. Compared to healthy subjects, DM2 patients have a lower ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG), a major endogenous antioxidant. In addition, malondialdehyde (MDA), a highly toxic by-product generated partially by lipid oxidation and ROS, leads to oxidative damage in the classical secondary targets of diabetes such as eyes, kidneys, nerves, and blood vessels. Islet cells of langerhans in the pancreas itself are the prime target, where the β-cell contains the lowest level of antioxidant enzyme activities compared to other tissues (Brownlee, 2001). A major manifestation of glucose toxicity in the β-cell is defective insulin gene expression, diminished insulin content, and insulin secretion due to defective glucose transporter protein (GLUT) or insulin receptor (Evans, 2007).

2.1.2 Dyslipidemia

Obesity is the main risk factor in the development of metabolic syndrome. The precise pathogenesis of diabetic dyslipidemia is not known, while obesity may be the main risk factor accompanied by increased levels of leptin and cytokines in the circulation, which modulates the β-cell function and their survival (Maedler *et al*., 2003). The combined elevation of cytokines such as IL-6 and IL-1β might affect lipid metabolism by acting on the liver to produce characteristic dyslipidemia in the metabolic syndrome, with high triglyceride and low HDL cholesterol levels as well as an increase in the concentration of small dense LDL-cholesterol. Likewise, the pro-inflammatory cytokine TNF-α reduces insulin sensitivity in the muscle tissue and stimulates hepatic lipogenesis, leading to hyperlipidemia (Franckhauser *et al*., 2008).

The lipid changes associated with diabetes mellitus attribute to increased free fatty acid (FFA) flux to insulin resistance (Roehrich *et al*., 2003; Donath *et al*., 2003). In diabetic dyslipidemia, it is also evident that NF-κB signaling is involved in the low-grade inflammation which occurs in the liver (Cnop *et al*., 2002). As we know, NF-κB is one of the most important regulators of pro-inflammatory gene expression, which regulates β-cells by inducing apoptosis or promoting their survival. Depending on the kinetics and mode of induction involved in the etiology of insulin resistance, this signaling node may influence peripheral insulin resistance via actions in myeloid cells (Maedler *et al*., 2004). Hence, this supports the role of NF-κB in causing insulin resistance and impairing insulin secretion with dyslipidemia in type 2 diabetes.

2.1.3 Inflammatory response

Recent evidence suggests that high levels of ROS and subsequent oxidative stress are the key contributors in the development of diabetic complications which weaken the antioxidant defences through an elevation in inflammation and inflammatory mediators by exerting major effects on signaling pathways, which further affects cellular metabolism and triggers a low-grade inflammatory reaction (Dominiczak, 2003). Lipid accumulation in adipose tissue and expansion of the fat mass in the liver initiate steatosis that promotes low-grade inflammation via activation of NF-κB (Arkan *et al*., 2005) and provokes an inflammatory process accompanied by local production and secretion of pro-inflammatory cytokines and chemokines (Hotamisligil *et al*., 1995; Jager *et al*., 2007).

It has been hypothesized that DM2 is a manifestation of an ongoing acute-phase response that is primarily characterized by alterations of the so-called acute-phase proteins such as the C-reactive protein (CRP) (Pickup and Crook, 1998; Pickup *et al*., 1997), with other cytokines that are central mediators of inflammatory reactions, such as IL-6, IL-1β or tumor necrosis factor α (TNF-α). It is well-established that cytokines operate as a network in stimulating the production of acute-phase proteins. For example, the effects of IL-6 on CRP synthesis largely depend on its interaction with IL-1β (Spranger *et al*., 2003). TNF-α reduces insulin sensitivity in smooth muscles and tissues, thereby stimulating hepatic lipogenesis (Franckhauser *et al*., 2008).

Investigations into the role of inflammatory mechanisms in diabetes and its complications are expected to provide insight into the processes and onset of progression in the disease. Such improved understanding of the inflammatory basis for diabetes may prove valuable in introducing novel approaches to treatment, alongside the currently used non-pharmacologic and pharmacological interventions.

2.2 Inflammatory cytokines and cancer

Cytokines are molecular messengers that allow the cells of the immune system to communicate with one another to generate a coordination, robust, but self-limited response to a target antigen. The mixture of cytokines that is produced in the tumor microenvironment has an important role in cancer pathogenesis. Cytokines that are released in response to infection, inflammation and immunity may act to inhibit tumour development and progression. Alternatively, cancer cells can respond to host-derived cytokines that promote growth, attenuate apoptosis and facilitate invasion and metastasis (Glenn, 2004).

Cancer is mediated through the inflammation process. In the inflammatory process, cytokines and pro-inflammatory cytokines mediate in the pathogenesis of carcinogenesis as well as tumor growth and spread; cytokines such as IL-1 and TNF-α induce chemokines that attract neutrophils. Neutrophils are the key players in the production of ROS and carcinogenesis which is induced by these cytokines and reflects in the type of genes they induce (Achyut and Yang, 2011).

Cytokines such as TNF-α, IL-1 and IL-6 are primarily pro-inflammatory which are responsible for the induction of adhesion molecules and metalloproteinases, both of which provide mechanisms for tumor invasion.

Up to 20% of all cancers arise in association with chronic inflammation, and most, if not all solid tumors, contain inflammatory infiltrates. Immune cells have a broad impact on tumor initiation, growth and progression which are mediated by proinflammatory cytokines (Yagi *et al*., 2002). Cytokines TNF-α, IL-1, IL-6 and IL-1β are master regulators of tumor-associated inflammation and tumorigenesis. This makes them attractive targets for adjuvant treatment in cancer. A more detailed understanding

of cytokine-tumor-cell interaction may provide opportunities for the development of a new plant-based cancer therapy.

2.3 Antioxidant defence

Antioxidants are substances that protect the body from damaging oxidation reaction by neutralizing free radicals and other ROS within the body through hindering the process of oxidation (Butkovic *et al*., 2004). Antioxidant defence involves several forms that include both enzymatic and non-enzymatic systems. The potent antioxidant activity exists in polyphenolics and may be the most important function, e.g. by scavenging or quenching free radicals, chelating metal ions, or inhibiting enzymatic systems responsible for free radical generation (Blaha *et al*. 2004; Dias *et al*. 2005).

The activities of polyphenolic compounds and their benefits in oxidative stress-related disorders have been widely studied. It is now widely accepted that dietary polyphenolics may play an important role in protecting the body against acute or chronic diseases such as diabetes, inflammation, cancer and cardiovascular diseases (Knekt *et al*. 2002; Karaca *et al*. 2006). Dietary polyphenols from fruits, vegetables and medicinal herbs possess wide therapeutic benefits in ameliorating diabetic complications. Flavonoids as phenolic compounds inhibit the polyol pathway and are also involved in preventing the overproduction of superoxide and in metal ion chelation processes (Abdallah *et al*., 2011; Boots *et al*., 2008). Thus, plants that are rich in flavonoids could be promising in the management of diabetes and its related complications.

The antioxidant action of flavonoids, the best described biological activity of this group of natural polyphenolic substances, is covered by a large number of studies (Amic *et al*., 2007; Bischoff, 2008). Phenolic and flavonoids as naringenin, kaemferol,

quercetin, and caffeic acid demonstrated divergent activities such as anti-diabetic, anti-oxidant, and anti-inflammatory activities with potential anticancer properties. (Jung *et al*., 2006; Ortiz-Andrade *et al*., 2007; Tsai *et al*., 2012; Zhang *et al*., 2009).

2.3.1 Antioxidant testing

Different antioxidants scavenge different free radicals and may be effective in different parts of the body. Antioxidant effectiveness is measured by monitoring the inhibition of oxidation of a suitable substrate. When the substrate is oxidized under a standard condition, the extent of oxidation (an end-point) is measured by chemical or biosensory methods.

2.3.2 DPPH assay

The measurement is based on the measurement of the scavenging activity of antioxidants towards the stable radical 2, 2-diphenyl-1-picryl-hydrazyl (DPPH). Antioxidants react with DPPH and are reduced to the DPPH-H; as a result, the absorbance decreases from the DPPH radical (purple) to the DPPH-H form (yellow). The level of discoloration indicates the scavenging potential of the antioxidant compounds (IH) in term of its hydrogen-donating ability. The discoloration of DPPH dissolved in methanol or ethanol is followed by monitoring the absorbance at 518 nm (Choi *et al*., 2002).

$$\mathbf{DPPH^{\cdot} + IH \longrightarrow DPPH - H + I^{\cdot}}$$

DPPH assay is a rapid, simple, inexpensive and reproducible assay that employed a stable radical to evaluate the scavenging ability of the natural product to act as free radical scavengers or hydrogen donars.

2.3.3 ORAC assay

ORAC stands for Oxygen Radical Absorbance Capacity method. The assay measures the oxidative degradation of the fluorescent molecule (either beta-phycoerythrin or fluorescein) after being mixed with free radical generators such as azo-initiator compounds. Azo-initiators are considered to produce the peroxyl radical by heating, which damages the fluorescent molecule and results in the loss of fluorescence. The fluorescent intensity decreases as the oxidative degeneration proceeds, and this intensity is typically recorded for 35 minutes after the addition of the azo-initiator (free radical generator). Antioxidants protect the fluorescent molecule from the oxidative degeneration. Curves of fluorescence intensity vs time are recorded, and the area under the curves, with and without the addition of an antioxidant, is calculated and compared to a standard curve generated using the antioxidant standard (Trolox) (Choi *et al*., 2002).

2.3.4 FRAP assay

FRAP stands for Ferric Reducing/Antioxidant Power method. This method measures the ability of antioxidants to reduce ferric iron. It is based on the reduction of the complex of ferric iron and 2,3,5-triphenyl-1,3,4- triaza-2-azoniacyclopenta-1,4-diene chloride (TPTZ) to the ferrous form at low pH. The reaction is non-specific, in that any half reaction that has lower redox potential than that of the ferric ferrous half reaction-under reaction conditions- will drive the ferrous (Fe III to Fe II) ion formation. This reduction is monitored by measuring the change in absorption at 593 nm. The change in absorbance is therefore directly related to the combined or “total” reducing power of the electron-donating antioxidants present in the reaction mixture (Benzie and Strain, 1996).

2.4 *In vitro* studies on cell lines

Cells are considered to be the basic 'building blocks' of living organisms. There is a great need for physiologically-relevant human or animal cell-based studies that can provide important biomedical and toxicity data. With the current growth in new technologies, knowledge, and methods for complex data analysis, it now appears feasible that they will be able to be reassembled into models that can better predict human cells with their capabilities of generation and degeneration. Some of the recent developments and trends in cell-based assays that are relevant to the future of toxicity testing are important (Swedlow and Platani., 2002). Cell-based bio-assays can play an important role in the evaluation of natural products and other medicinal plants as an initial tool for screening, or as follow-up to animal or human studies in diabetes, inflammation and cancer research.

2.4.1 Pancreatic β-cells

In diabetes research, insulin-secreting cell lines have been developed in an attempt to establish and retain the characteristic features of β-cells. These cell lines are transformed using different techniques such as irradiation, viral transformation, and transgenic technology. They can therefore be different from primary β-cells in terms of their behaviour and responsiveness to insulin secretagogues. The most widely used β-cells lines are β-TC6, RINm5F, HIT-T15, MIN6, INS-1, and BRIN-BD11 cells (Poitout *et al*., 1995).

One such β-TC6 (insulinoma cell line derived from transgenic mice) is developed with higher hexokinase activity, resulting in an insulin secretory response to subphysiological glucose levels (Leira *et al*., 2002; Poitout *et al*., 1995). The advantage of this β-TC6 cell lines is that it can be used in the rapid analysis of cellular viability &

proliferation, insulin secretion, as well as glucose uptake with glucose transporter protein expression (Yamada *et al*., 2007; Zou *et al*., 2005).

2.4.2 Murine macrophage cells

Murine macrophage RAW 264.7 cells are widely used to determine the inflammatory pattern of the cytokines and chemokines. In this cell line, cytokine levels are triggered by using a certain stimulating agent to induce inflammatory response (Huo *et al*., 2012). Such an activator of macrophage is a Lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, which initiates Toll-like receptor 4 (TLR4)-associated innate immune response by triggering serial signal transduction events which lead to the activation of NF-κB and mitogen-activated protein kinases (MAPKs) (Dziarski *et al*., 2000; Van Snick, 1990). The activation of these proteins leads to the production of various inflammatory cytokines TNF-α, IL-1β, IL-6 and NO (Zhang and Dong, 2005; Medzhitov and Janeway, 1997). Thus, these cytokines are recognized as excellent biomarkers for inflammation in order to study inflammatory response within the living system (Huang *et al*., 2012; Yang *et al*., 2011).

2.4.3 Cancer cells

For decades, human immortal cancer cell lines have constituted an accessible, easily usable set of biological models with which to investigate cancer biology and explore the potential efficacy of anticancer agents. In cancer research, a wide range of human cancer and normal cell lines are utilized to study the efficacy of several natural products. Cancer cells are characterized by an uncontrolled, uncoordinated and undesirable cell division. Unlike normal cells, cancer cells continue to grow and divide for their whole lives, replicating into more and more harmful cells.

Cancer cell lines allow scientists to conduct studies under reasonable conditions in the lab to study the communication between a tumor cell and a host cell. In our study,

we have selected lung cancer (A549), prostate cancer (PC-3), breast cancer (MCF-7), and normal hepatic (WRL-68) cell lines to evaluate the potential of our extracts/fractions in malignant neoplasm, which is a broad group of various diseases, all involving unregulated cell growth and can be studied for cell proliferation and apoptosis.

2.4.4 Role of NF-κB in research

NF-κB is a transcription factor involved in the gene encoding and regulation which may constitute immune and inflammatory responses by activating cell survival, signalling, proliferation, angiogenesis, and invasion, which are key features of the malignant phenotype (Baldwin, 1996; Haefner, 2002). NF-κB is believed to play an important role in the regulation of insulin signalling pathway and inflammatory response associated with cancer therapy in copious inflammatory and cancer-related ailments and has developed as a foremost target in drug discovery (Collins *et al*., 1995; Das and White, 1997).

2.5 *In vivo* studies on animal models

Studies using biological systems help us to understand the detailed mechanism of the targeted candidate for further drug development. Preclinical **s**tudies on animal model play an important role in discovering the efficacy and safety of the candidate. In preliminary screening, normal animals are sensitive enough to be used in determining the effectiveness and safety of natural products (Hoa *et al*., 2009). Target animal models are developed in normal animals with stimulating agent or substances for detailed studies.

2.5.1 Type 1 diabetes in animals

In diabetes research, diabetic rat models are mostly utilized to determine the potential of natural products for the management of diabetes. Chemically-induced diabetes in animals is considered as a conventional model of type 1 or insulin dependent diabetes mellitus (IDDM) because the chemical selectively destructs β-cells of the pancreas, leading to a decrease in insulin synthesis (Ahmed, 2006; Kundusen *et al*., 2011). However, the incomplete loss of β-cells mass and persistent hyperglycemia can also result in insulin resistance which is a characteristic feature of non-insulin-dependent diabetes mellitus (NIDDM) (Shima *et al*., 1998).

Streptozotocin (STZ) is the cytotoxic substance used conventionally to produce diabetes and hyperglycemia in experimental animals by selectively destroying β-cells (Masiello *et al*., 1998). STZ is a 1-methyl-l-nitrosourea attached to the carbon- 2 position of glucose that causes β-cell necrosis and induces ‘‘experimental diabetes’’ in animals (Thulesen *et al*., 1997). The glucose moiety of STZ allows preferential uptake of STZ into β-cells, probably via the glucose transporter-2 (GLUT-2) and causes alkylation of DNA (Jin *et al*., 2009). Because STZ is an alkylating agent, it causes DNA strand breaks that induce the activation of poly-ADP-ribose synthetase followed by lethal nicotinamide adenine dinucleotide (NAD) depletion (Viraj and Szabo., 2002).

This NAD depletion is also mediated by cytokine, T-helper cell and macrophages. Activated macrophages produce a variety of free radicals, nitric oxide (NO) as well as IL-6 and IL-1β, which have been found to activate the inducible form of nitric oxide synthase (iNOS), thus causing an increased production of NO within the β-cell. The potential problem with STZ due to its toxic effects is not restricted to pancreatic β-cells, since it may also cause renal injury, oxidative stress, inflammation, and endothelial dysfunction (Hyun *et al*., 2010).

2.5.2 Type 2 diabetes in animals

Type 2 diabetes in animals is established by a suitable dose of nicotinamide (NA) prior to STZ administration, which exerts a partial protection against the cytotoxic effects of STZ (Masiello *et al*., 1998; Yang and Wright, 2002). Nicotinamide (vitamin B3), a water-soluble vitamin and a poly-ADP-ribose synthetase inhibitor, protects the functionality of β-cells by protecting the level of NAD and proinsulin from depleting, which improves the energy status in ischemic tissues and exhibits antioxidant effects. This leads to metabolic improvements through the inhibition of the apoptosis of β-cell mass by partially reversing the inhibition of insulin secretion to prevent the complete aggravation of β-cells destruction following the administration of β-cell toxins, STZ (Shima *et al*., 1998).

This established a new experimental diabetic syndrome in rats that appears closer to human type 2 diabetes than other available models (e.g. neonatally STZ-injected rats, GK rats). This condition contributes a number of features similar to DM2, and is exemplified by stable hyperglycemia, glucose intolerance, and significantly altered glucose-stimulated insulin secretion (Like and Rossini., 1976).

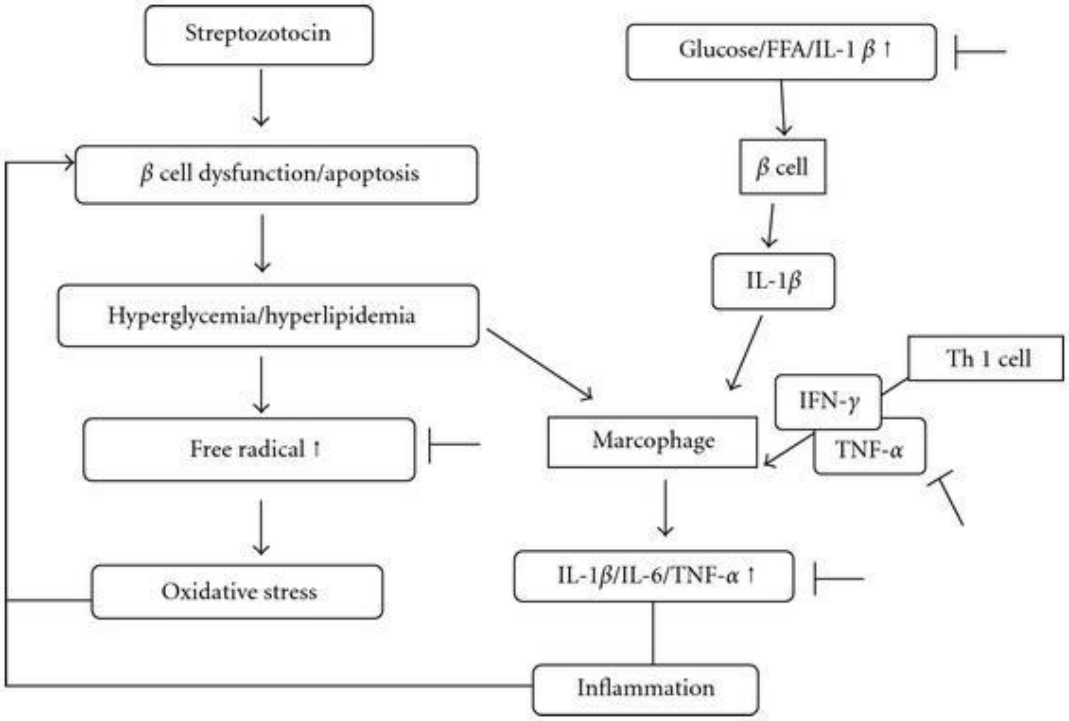

Figure 2.1**:** Hypothetical diagram displaying STZ-induced diabetic complications.

When normal animals are induced by STZ, they develop hyperglycemia and associated complications such as hyperlipidemia, oxidative stress and inflammatory response. These elevated responses may be primary targets for the type 2 diabetes (DM2) research (Shi *et al*., 2011).

This study model through preclinical approach may help us to evaluate whether the samples can further pave the way for the development of medication, particularly for the management of diabetic conditions and its complications that arise during this metabolic syndrome.

2.5.3 Inflammatory response in animals

Inflammatory response on targeted cytokines can be achieved by stimulating animals with certain doses of LPS (Achoui *et al.*, 2010). The mechanism by which LPS induces these events is only partly understood. At physiological concentrations, LPS binds to the serum protein LPS-binding protein (LBP). This complex then interacts with glycosylphosphatidylinositol-linked protein (CD14) on macrophages or neutrophils

(Palsson-McDermott and O'Neill, 2004). CD14 lacks an intracellular domain, and when LPS-LBP complex binds to CD14, it results in rapid phosphorylation of various proteins on tyrosyl residues, which in turn provokes proinflammatory cytokines (Remick *et al*., 2000). During this process, inflammatory cytokine TNF-α is commonly released by macrophages and plays a central role in initiating and sustaining the inflammatory response; this can cause severe tissue damage, septic shock, atherosclerosis and systemic inflammatory response syndrome (Chiang *et al*., 2012; Hseu *et al*., 2005; Garlanda *et al*., 2007).

This LPS-induced inflammatory response of cytokines would help us to determine the effectiveness of the samples against different cytokines which were activated.

2.6 Plants Selection

The rationale behind the selection of the following plant species are their traditional use by the villagers and traditional healers. Plants selected were *Centratherum anthelminticum* L., *Parthenium hysterophorus* L., *Xanthium strumarium* L., and *Vicoa indica* Cass, which are all from the Asteraceae family, together with five others from different families: *Woodfordia fruticosa* Kurz (Lythraceae), *Sida acuta* Burm F. (Malvaceae), *Vanda tessellata* Roxb (Orchidaceae), *Madhuca indica* (Sapotaceae) and *Cissus quadrangularis* (Vitaceae). Three others are from the same family (Combretaceae) and the same genus *Terminalia* such as *T. arjuna*, *T. bellerica*, and *T. chebula*.

Out of all these plants, *Centratherum anthelminticum* seeds were discovered to be the most prominent in terms of their hypoglycemic and antioxidant activities. Therefore, *Centratherum anthelminticum* seeds were further selected in our study for

the purpose of detailed pharmacological evaluations on diabetes, inflammatory response and cancer.

2.7 *Centratherum anthelminticum* (L.) Kuntze

Centratherum anthelminticum (L) Kuntze (Family: Asteraceae) (Synonyms: *Vernonia anthelmintica)* is an erect, pubescent annual herb found widely in the Indian sub-continent. The stem grows to a height of 90 cm. The leaves are elliptical, nearly 9 cm long and 3.5 cm wide with an acute apex and tapering base. The margins are coarsely serrated and both sides of the leaves are pubescent. The flowers are purple or violet, homogenous, solitary, axillary or terminal heads with a linear bract at the top of the peduncle. Each flower head consists of 30-40 minute branches. The fruits are small, cylindrical and hairy with 10 narrow ridges. The seeds of the plant have a sharp, bitter taste and possess wide therapeutic applications (Rastogi and Mehrotra, 1995).

(A)

(B)

(C)

Figure 2.2**:** Picture of *Centratherum anthelminticum* (L.) Kuntze
(A) whole plant, (B) flowering twigs (C) dried seeds.

2.7.1 Chemical Constituents

Table 2.1: Chemical constituents from *Centratherum anthelminticum* seeds reported in literature

Compounds	**References**
Glycosides, carbohydrates, phenolic compounds, tannins, flavonoids, proteins, saponins, sterols, lipids and fats	Bhatia *et al*., 2008
Flavonoids : 2',3,4,4'-tetrahydroxychalcone (butein), 5,6,7,4'-tetrahydroxyflavone (scutellarein) and 7,3',4'-trihydroxydihydroflavone (butin).	Tian *et al*., 2004
Sterols: 4 alpha-methylvernosterol, vernosterol, avernosterol .	Akihisa *et al*., 1992
glycosylated triterpene, saponin: hederagenin and six sugar residues forming two glycosyl chains.	Mehta *et al*., 2004
Steroids : (24alpha/R)-stigmasta-7-en-3-one, 24 (alpha/R)-stigmasta-7, 9(11)-dien-3-one, 24(alpha/S)-stigmasta-5, 22-dien-3ß-ol and stigmasta-7, 22-dien-3ß-ol	Mehta *et al*., 2005
Saponins: 3-O-[beta-D-glucopyranosyl-(1-->2)-alpha-L-rhamnopyranosyl-(1--> 2)-alpha-L-arabinopyranosyl]-28-O-[beta-D-xylopyranosyl-(1-->4)-alpha-L-rhamnopyranosyl-(1-->3)-beta-D-glucopyranosyl]-23-hydroxyolean-12-en-28-oic acid and 3-O-[beta-D-glucopyranosyl-(1 --> 2)-alpha-L-rhamnopyranosyl-(1 -->2)-alpha-L-arabinopyranosyl]-28-O-[beta-D-glucoyranosyl-(1-->3)-beta-D glucopyranosyl]-23-hydroxyolean-12-en-28-oic acid	Mehta *et al*., 2010

2.7.2 Pharmacological activities

Centratherum anthelminticum seeds are used in Ayurvedic preparations due to their febrifugal, anthelmintic, antiphlegmatic, and diuretics as well as their digestive stimulant properties (Kirtikar and Basu, 1987).

Singh *et al* (1985) demonstrated *in vitro* anthelmintic activity on the alcoholic extract of *Centratherum anthelminticum* seeds against *Fasciolopsis buski*, *Ascaris lumbricoides* and *Hymenolepis nana* worms. Next, Sharma and Mehta (1991) determined *in vitro* antimicrobial effects of *Centratherum anthelminticum* seeds. Further, Singhal *et al* (1992) reported antifilarial activities on *Setaria cervi* in the aqueous and alcoholic extract of *Centratherum anthelminticum* seeds.

Iqbal *et al* (2006) demonstrated *in vivo* anthelmintic activity of *Centratherum anthelminticum* seeds in sheep naturally infected with gastrointestinal nematodes. Furthermore, Nisha *et al* (2007) showed *in vitro* macrofilaricidal activity of *Centratherum anthelminticum* seeds against adult *Setaria digitata*, the cattle filarial worm.

Koti and Purnima (2008) reported a potent diuretic and electrolyte excretion activities in the chloroform and alcohol extracts of *C. anthelminticum* seeds. Srivastava *et al* (2008) observed that the petroleum ether extract of *Centratherum anthelminticum* fruits exhibited significant larvicidal activity against the vector of malaria, *Anopheles stephensi*.

Purnima *et al* (2009) established *in vivo* analgesic and antipyretic effects in the petroleum ether and alcohol extracts of *Centratherum anthelminticum* seeds. The same co-workers in the year 2010 reported antiinflammatory activity in the *in vivo* models of acute and subacute inflammation. The results showed that these extracts exert

antiinflammatory activity through prostaglandin inhibition by reducing myeloperoxidase transduction.

Ani and Naidu (2011) determined the antioxidant potential of *Centratherum anthelminticum* seeds in certain *in vitro* models. Moreover, Faheem and Koay (2011) reviewed the studies on the chemical constituents and pharmacology of *Centratherum anthelminticum.* Recently, Himanshu *et al* (2012) demonstrated wound healing properties in the *in vivo* experiment on the aquous methanolic extract of *Centratherum anthelminticum* seeds.

2.7.3 Antidiabetic activity

Ani and Naidu (2008) showed antidiabetic effects of the phenolic compounds fractionated from *Centratherum anthelminticum* seeds on the rat intestinal α-glucosidases, human salivary α-amylase and postprandial hyperglycemia in rats. The results showed that the polyphenolic components significantly inhibited postprandial hyperglycemia in rats by altering α-amylase and α-glucosidase activity.

Next, Bhatia *et al* (2008) established antidiabetic activities on the aqueous extract of *Centratherum anthelminticum* seeds in alloxan-induced diabetic rats. The results displayed that dose-dependent administration of the extract reduced plasma glucose and the results were comparable with the oral hypoglycemic drug glibenclamide.

Shah *et al* (2008) showed antihyperglycemic, antihyperlipidemic and antioxidant potential of *Centratherum anthelminticum* seeds on STZ-induced diabetic rats in a short term study model. Next, Fatima *et al* (2010) demonstrated antidiabetic and antihyperlipidemic activities of ethyl-acetate:isopropanol (1:1) fraction of *Vernonia anthelmintica* seeds in STZ-induced diabetic rats, without any toxicity in normal rats.

2.7.4 Antiinflammatory activity

To date, there are no scientific reports on the antiinflammatory responses of the extract/fractions of the *Centratherum anthelminticum* seeds on the targeted cytokines in the cell-based *in vitro* and *in vivo* study models.

2.7.5 Anticancer activity

Thus far, *Centratherum anthelminticum* has not been evaluated for its anticancer effects and hence, there are no reports on its activities on any type of cancer cells.

2.7.6 Knowledge (study) gap

To date, there are no reports of mechanistic studies on the antidiabetic effects of *Centratherum anthelminticum* seeds on the pancreatic β-cells for cellular viability, glucose uptake, insulin secretion, glucose transporter protein expression as well as on the NF-κB translocation effects in ROS-induced oxidative stress. Moreover, there are no indications on the *in vivo* type 1 and type 2 diabetic rat models to elucidate the role of this plant seeds in diabetic conditions. In addition, there are no studies on type 2 diabetic animals to determine the protective and toxic effects of the selected extract/fraction when administered for a long term period of 12-weeks on the DM2 complications such as oxidative stress, dyslipidemia and elevated pro-inflammatory cytokines.

Other pleotropic biological activities on inflammation and cancer are yet to be investigated on this plant seeds, which include activities such as inflammatory responses of cytokine TNF-α in the *in vitro* and *in vivo* study models as well as on certain targeted cancer cell lines such as A549, PC-3, MCF-7, and WRL-68 cells, alongwith mechanistic studies on the targeted cancer cells.

CHAPTER 3

METHODS, RESULTS AND DISCUSSION

3.1 List of publications

Screening for hypoglycemic activity on the leaf extracts of nine medicinal plants: *in-vivo* evaluation

Aditya Arya., Mahmood Ameen Abdullah., Batoul Sadat Haerian., & Mustafa Ali Mohd. (2012). Screening for hypoglycemic activity on the leaf extracts of nine medicinal plants: *in-vivo* evaluation. *E-Journal of Chemistry, 9*(3) 1196-1205. (**ISI Journal, Impact Factor: 0.716**)

Antioxidant and hypoglycemic activities of leaf extracts of three popular *Terminalia* species

Aditya Arya., Shaik Nyamathulla., Mohamed Ibrahim Noordin., & Mustafa Ali Mohd. (2012). Antioxidant and hypoglycemic activities of leaf extracts of three popular *Terminalia* species. *E-Journal of Chemistry, 9*(2) 883-892. (**ISI Journal, Impact Factor: 0.716**)

Anti-diabetic effects of *Centratherum anthelminticum* seeds methanolic fraction on pancreatic cells, β-TC6 and its alleviating role in type 2 diabetic rats

Aditya Arya., Chung Yeng Looi., Shiau Chuen Cheah., Mohd Rais Mustafa., & Mustafa Ali Mohd. (2012). Anti-diabetic effects of *Centratherum anthelminticum* seeds methanolic fraction on pancreatic cells, β-TC6 and its alleviating role in type 2 diabetic rats. *Journal of Ethnopharmacology, 144*(1) 22-32. (**ISI Journal, Impact Factor: 3.014**)

The methanolic fraction of *Centratherum anthelminticum* seed downregulates pro-inflammatory cytokines, oxidative stress, and hyperglycemia in STZ-nicotinamide-induced type 2 diabetic rats

Aditya Arya., Shiau Chuen Cheah., Chung Yeng Looi., Hairin Taha., Mohd Rais Mustafa., & Mustafa Ali Mohd. (2012). The methanolic fraction of *Centratherum anthelminticum* seed downregulates pro-inflammatory cytokines, oxidative stress, and hyperglycemia in STZ-nicotinamide-induced type 2 diabetic rats. *Food and Chemical Toxicology, 50*(11) 4209-4220. (**ISI Journal, Impact Factor: 2.999**)

Chloroform fraction of *Centratherum anthelminticum* (L.) seed inhibits tumor necrosis factor alpha and exhibits pleotropic bioactivities: inhibitory role in human tumor cells

Aditya Arya., Mouna Achoui., Shiau Chuen Cheah., Siddig Ibrahim Abdelwahab., Putri Narrima., Syam Mohan., Mohd Rais Mustafa., & Mustafa Ali Mohd. (2012). Chloroform fraction of *Centratherum anthelminticum* (L.) seed inhibits tumor necrosis factor alpha and exhibits pleotropic bioactivities: inhibitory role in human tumor cells. *Evidence-Based Complementary and Alternative Medicine*, doi:10.1155/2012/627256. (**ISI Journal, Impact Factor: 4.743**)

3.2 An overview of the research approach employed in this study

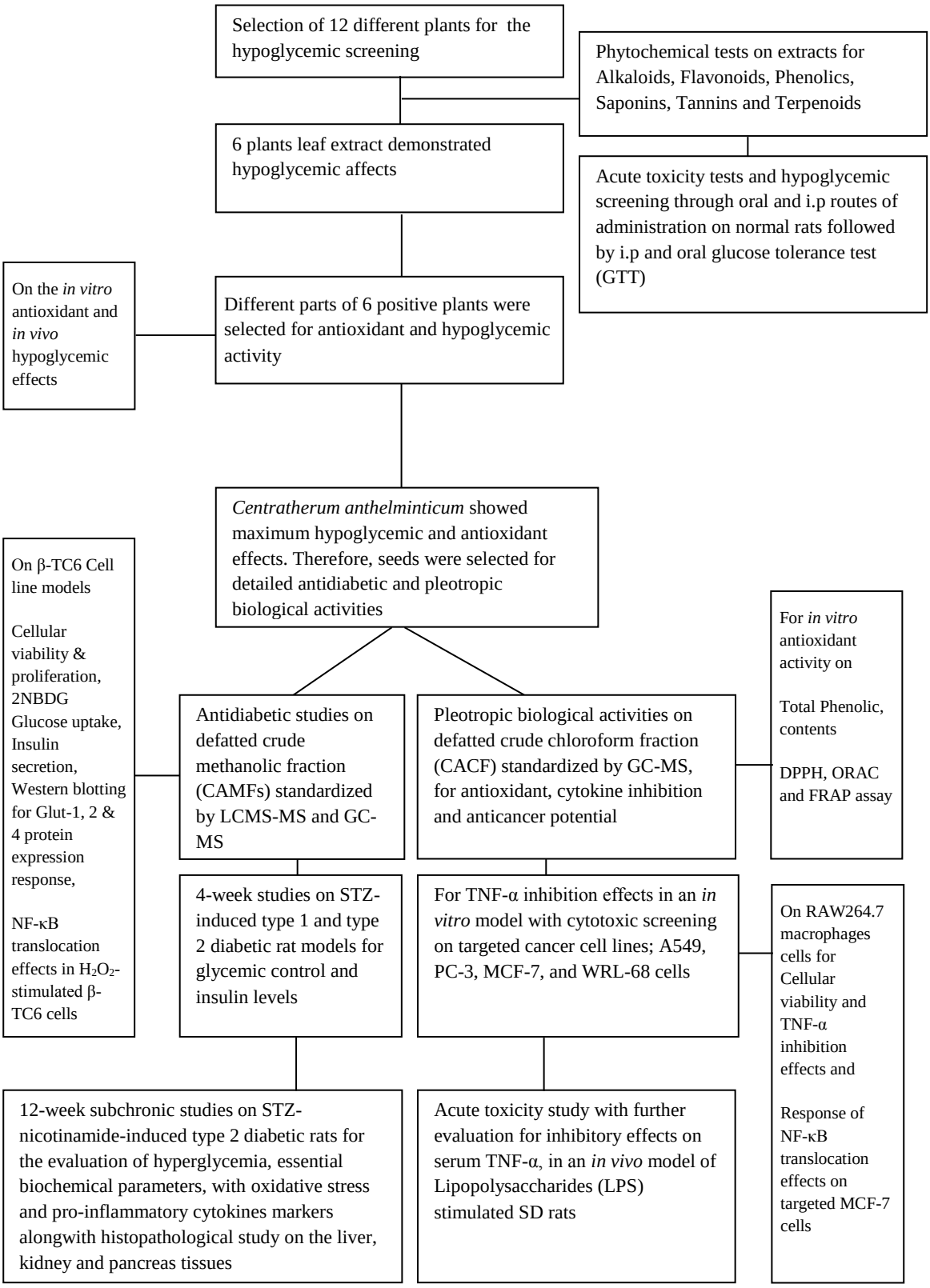

Figure 3.1: An overview of the research approach employed in this study. For detailed methodology, see published papers in the section 3.3

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Screening for Hypoglycemic Activity on The Leaf Extracts of Nine Medicinal Plants: *In-Vivo* Evaluation

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Abstract: The traditional use of certain plants by the tribal community in central India involves using the young leaves for several ailments, including hyperglycaemia; this study was performed to evaluate the effects of the leaf extracts from 9 such plants in the management of diabetes. Initially, hypoglycemic screening was performed on normal rats whose blood glucose levels were measured before and after oral or intraperitoneal (i.p.) administration of the extracts at different periods. The plants were screened at doses of 250 mg/kg i.p. or 500 mg/kg orally. Of these, only *Centratherum anthelminticum* (Asteraceae), *Cissus quadrangularis* (Vitaceae), and *Woodfordia fruticosa Kurz* (Lythraceae) significantly reduced postprandial blood glucose levels in normal glycemic rats (P <0.001), with slight reductions effected by *Sida acuta Burm F.* (P = 0.002) and *Parthenium hysterophorus L.* (P = 0.017). The extracts that reduced postprandial blood glucose levels both orally and i.p. in the hypoglycemic screening tests were evaluated for glucose challenge in glucose tolerance tests with i.p and oral administration in overnight-fasted normal rats. The results of these tests potentiate the screening data in the management of diabetes mellitus, which requires further studies on the plants that yielded positive results to determine the active compounds in the different plant parts that are responsible for the activity.

Keywords: Hypoglycemic activity, Traditional medicine, Herbals, Glucose tolerance test,*Centratherum anthelminticum*, *Cissus quadrangularis*, *Woodfordia fruticosa*.

Introduction

Diabetes mellitus (DM) has attained pestilencein the current century. It is the most prevalent disease in the world affecting 7% of population, or 285 million people worldwide. It is predicted that this number will exceed to 435 million in 2030.[1] Approximately, 90-95% of patients with diabetes have type 2 diabetes (T2D) or non-insulin dependent diabetes mellitus. T2D is accounting for a combination of insulin resistance and an adequate compensatory insulin secretory response.[2] Oral hypoglycaemics such as biguanides, sulfonylureas, and thiazolidinediones are available for the treatment of type 2 diabetes, but can also cause adverse effects and be ineffective against some long-term diabetic complications. The World Health Organization recommends the use of traditional and plant based medicines for the management of diabetes mellitus.[3] Herbal medicine is an alternative method for the treatment of diabetes due to their perceived effectiveness, safety, affordability, and acceptability, with minimal side effects in clinical experience, and relatively low cost.[4]

More than 1200 plants are used in traditional medicine for their alleged hypoglycemic activity. Numerous studies have reported the anti-diabetic activity of plants.[5] In the present study, the rationale for selecting the 9 plants studied was their traditional use by tribal people for conditions involving frequent urination and elevated blood glucose levels with symptoms such as weakness, mouth dryness, and conditions associated and arising from diabetes. The tribes are from villages in the Betul and Hoshangabad districts, which are surrounded by the Satpuda forest ranges, and located in the state of Madhya Pradesh, India. In these districts, villagers consume decoctions from the young leaves of these plants early in the morning for the treatment of diabetes as well as for other ailments. The treatment is particularly common and popular in tribes of the Gond community, where the plants are cultivated or found in abundance around their farms or in the jungles. The plants tested were *Centratherum anthelminticum*, *Parthenium hysterophorus L.*, *Xanthium strumarium L.*, and *Vicoa indica Cass*, which areall from the Asteraceae family, together with five others from different families: *Woodfordia fruticosa Kurz* (Lythraceae), *Sida acuta Burm F.* (Malvaceae), *Vanda tessellata* (*Roxb.*) (Orchidaceae), *Madhuca indica* (Sapotaceae), and *Cissus quadrangularis* (Vitaceae). There are several reports on the antidiabetic effects of different parts of *C. anthelminticum*, *Parthenium hysterophorus* L., *Cissus quadrangularis*, and *Woodfordia fruticosa Kurz*.[6-11]The purpose of this study was to investigate and evaluate the potential of these plants leaves in suppressing elevated blood glucose levels in normoglycaemic and glucose-induced hyperglycaemic rats through the intraperitoneal (i.p.) and oral routes of administration.

Materials and Methods

Plant Materials and Preparation of Plant Extracts

Fresh leaves from the nine plants were collected from Amritum Bio-Botanica Herbs Research Laboratory Pvt. Ltd., in Jogli, Madhya Pradesh, India, authenticated by the company's quality control department, dried in maintained shade in a greenhouse for few days, ground into coarse powder, and then brought to the Department of Pharmacology in the Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia, for further study. The dried, powdered samples were extracted with water: ethanol (80:20)using a soxhlet extractor, subsequently the obtained crude extracts were successively defatted with hexane and chloroform, before being extracted with methanol. The resulting methanol extracts were dried (evaporated) using a rotary evaporator (Rotavapor, Buchi) under reduced pressure at

40°C to yield a final methanolic extract, which was further stored at -20°C prior to use. Before administration to the rats, each extract was diluted with water and filtered.

Preliminary Phytochemical Screening

A phytochemical screening test for alkaloids, flavonoids, saponins, phenols, tannins, and terpenoids using standard procedures was performed on the defatted leaf methanolic extracts to identify their chemical constituents.[12]

Animals and Treatment

Altogether, 400 Sprague-Dawley rats weighing (150–200 g) were procured from the Animal Care Unit, UMMC (University Malaya Medical Centre), Kuala Lumpur, Malaysia, and were acclimatized to laboratory conditions for 1 week. Rats were housed in polypropylene cages (6–7 rats/cage) under an ambient temperature of 23 ± 3°C and 40–65% relative humidity with 12 h light and dark cycles. They were maintained on a standard pellet diet and water *ad libitum*. Guidelines of the Guide for the Care and Use of Laboratory Animals were strictly followed throughout the study. The experimental protocol was approved by the UMMC Animal Ethics Committee (No: FAR/10/11/2008/AA(R)).

Acclimatized animals were divided into 2 sections, one for acute toxicity study, other section for hypoglycemic screening. Male animals were chosen for hypoglycemic screening test and allocated a carbohydrate-rich diet for 2 weeks before the experiments were started. Animals with postprandial blood glucose levels of 8–10 mmol/L were used for the screening test. Animals with either sex were selected for acute toxicity studies.

Acute Oral and Intraperitoneal Toxicity Studies

The oral and intraperitoneal acute toxicity tests of the extracts were determined in accordance with OECD (Organization for Economic Co-operation and Development) guidelines. The animals were starved overnight and divided into 36 groups (n = 6) and were injected intraperitoneally with extracts doses ranging 25–2500 mg/kg body weight and were fed with doses ranging 50–5000 mg/kg body weight. The rats were observed continuously for 2 h to assess their behavioural, neurological, and autonomic profiles and after 24 and 72 h to identify lethality.

Determination of Postprandial Blood Glucose Levels in Normoglycaemic Rats

Screening for hypoglycemic activity of the extracts was carried out by i.p and oral (250mg/kg and 500mg/kg) administration based on volume (2 ml/200 g body weight). Normal control animals received NaCl 0.9% while positive control animals were administered with insulin (0.5 U/kg) i.p. or glibenclamide (50 mg/kg) given orally.

Blood samples were collected from the tail by snipping, and blood glucose concentrations were determined in non-fasting conditions at different times after the above treatments by a glucose oxidase-peroxidase enzymatic method using a standardized glucometer (Accu-Check Performa, Roche Diagnostic Germany). An extract was deemed to have a hypoglycemic effect if the blood glucose levels of the test groups decreased significantly compared to that of the normal control group.

Glucose Challenge (Glucose Tolerance Test)

Intraperitoneal Glucose Tolerance Test (IPGTT) and Oral Glucose Tolerance Test (OGTT) were used to evaluate the effectiveness of the leaf extracts that had demonstrated hypoglycemic potential in the screening tests. Extracts (250 mg/kg or 500 mg/kg) were administered to overnight-fasted rats in doses of 2.0 ml/200 g (the control group was given NaCl 0.9%). Blood glucose concentrations were measured before and at 120 and 210 min, after i.p and oral extracts administration respectively. After this time point, oral glucose (3

g/kg) was administered and blood glucose levels were measured at 30, 60, and 120 min in the IPGTT and at 60, 120, and 180 min in the OGTT.

Statistical Analysis

The results were calculated as mean ± SEM and comparison of the data was carried out by 1-way and 2-way repeated measures ANOVA tests where appropriate (one factor repeated), with all pair-wise multiple comparison procedures (Student–Newman–Keuls method). Significance was considered to be $P<0.001$.

Results and Discussion

Plants Extract Phytochemical Constituents

Preliminary phytochemical analysis of methanolic extracts from the leaves of nine plants revealed the presence of flavonoids in all, except *Sida acuta Burm F.* and *Vanda tessellata* (Table 1). Similarly, phenolic and saponin compounds were present in all the leaves, while tannins were only absent in the *Sida acuta Burm F.* and *Vicoa indica* extracts. All the extracts, except that from *Madhuca indica*tested positive for alkaloids. Amongst the leaves, terpenoids were not seen in *Vanda tessellata (Roxb*.), *Parthenium hysterophorus L*., and *Sida acuta Burm F*. extracts.

Table 1. Phytochemical constituents of methanolic leaf extracts.

Plants	Parts Used	Compounds					
		Alkaloids	Flavonoids	Phenols	Saponins	Tannins	Terpenoids
Centratherum anthelminticum	Leaves	+	++	++	+	++	+
Cissus quadrangularis	Leaves	+	+	+	+	+	++
Woodfordia fruticosa Kurz	Leaves	+	+	+	+	+	+
Madhuca indica	Leaves	NA	+	+	+	+	+
Vicoa indica Cass	Leaves	+	+	+	+	NA	+
Vanda tessellata (Roxb.)	Leaves	+	NA	+	+	+	NA
Parthenium hysterophorus L.	Leaves	+	+	+	+	+	NA
Sida acuta Burm F.	Leaves	+	NA	+	+	NA	NA
Xanthium strumarium L.	Leaves	+	+	+	+	+	+

Acute Oral and Intraperitoneal Toxicity Studies

The acute oral and intraperitoneal toxicity studies demonstrated that the methanolic extracts from all of the selected plants leaf, other than *Parthenium hysterophorus L*., were non-toxic up to a dose of 2500mg/kg. For *Parthenium hysterophorus L*. the LD_{50} obtained via i.p administration was 2500 mg/kg. Based on this finding, doses with 250mg/kg and 500mg/kg were chosen as the maximum dose for further experiments involving administration by the i.p and oral routes respectively.

Hypoglycemic Activity of Plants Leaf Extracts by i.p. and Oral Routes

The results of the hypoglycemic activity of the leaf extracts on normal glycemic rats administered through the i.p. and oral routes are shown in Tables2 and 3. Accordingly, after i.p. and oral administration of NaCl 0.9% to normal control rats, the blood glucose levels did not change within the different time intervals. Administration of i.p. insulin (0.5 U/kg) significantly reduced blood glucose levels for 1–2 hours, then increased it again. A similar finding was observed following oral administration of glibenclamide (50 mg/kg), but the level of blood glucose returned back only after 6 h (P<0.001) for all when compared to time 0 and normal control group at the same times. Out of the nine plants, three (*C. anthelminticum*, *Cissus quadrangularis*, and *Woodfordia fruticosa Kurz*) significantly altered blood glucose levels (P<0.001), while four (*Madhuca indica.*, *Vicoa indica Cass*, *Vanda tessellata* (*Roxb*.), and *Xanthium strumarium L.*) did not.

Intraperitoneal administration of *C. anthelminticum*, *Cissus quadrangularis*, and *Woodfordia fruticosa Kurz*extracts at 250 mg/kg dose produced various outcomes, the blood glucose levels of rats decreased from 9.3 ± 0.11, 8.8 ± 0.19, and 9.1 ± 0.20 mmol/L, respectively to 4.9 ± 0.13, 5.6 ± 0.15, and 5.2 ± 0.18 mmol/L after the first hour. This reduction continued by the second hour to 4.6 ± 0.23, 5.3 ± 0.13, and 5.1 ± 0.16 mmol/L, respectively. Finally, unlike *C. anthelminticum* (4.3 ± 0.15 mmol/L after 3 h), a slight increase in blood glucose levels was observed with *Cissus quadrangularis* and *Woodfordia fruticosa Kurz* extracts after the third hour (5.5 ± 0.19 and 5.3 ± 0.13 mmol/L, respectively). A significant difference in blood glucose levels was observed after the first, second, and third hours following i.p. administration of *C. anthelminticum*, *Cissus quadrangularis*, and *Woodfordia fruticosa Kurz* extracts (P<0.001) when compared to that of the normal control.

Table 2. Hypoglycemic activity of leaf extract by i.p. route in normal rats.

Group	Blood glucose levels(mmol/L)at different time points (h)				
	Time (mean ± SEM)				
	0h	1h	2h	3h	P-value
Normal control (NaCl0.9%)	8.9 ± 0.09	9.0 ± 0.07	8.8 ± 0.24	8.8 ± 0.03	0.856
Insulin 0.5 U/kg	8.8 ± 0.14	4.4 ± 0.19*	4.3 ± 0.11*	5.0 ± 0.07*	<0.001
Centratherum anthelminticum	9.3 ± 0.11	4.9 ± 0.13*	4.6 ± 0.23*	4.3 ± 0.15*	<0.001
Cissus quadrangularis	8.8 ± 0.19	5.6 ± 0.15*	5.3 ± 0.13*	5.5 ± 0.19*	<0.001
Woodfordia fruticosa Kurz	9.1 ± 0.20	5.2 ± 0.18*	5.1 ± 0.16*	5.3 ± 0.15*	<0.001
Madhuca indica	8.9 ± 0.12	9.0 ± 0.16	8.7 ± 0.21	9.1 ± 0.13	0.420
Vicoa indica Cass	9.2 ± 0.10	9.0 ± 0.08	8.8 ± 0.21	9.0 ± 0.20	0.443
Vanda tessellata (Roxb.)	8.7 ± 0.19	8.5 ± 0.19	8.8 ± 0.17	8.7 ± 0.10	0.765
Parthenium hysterophorus L.	8.8 ± 0.10	8.9 ± 0.11	8.5 ± 0.14	8.6 ± 0.10	0.072
Sida acuta Burm F.	8.6 ± 0.13	8.4 ± 0.11	8.2 ± 0.18	8.7 ± 0.74	0.065
Xanthium strumarium L.	8.8 ± 0.19	9.0 ± 0.27	8.9 ± 0.12	8.8 ± 0.17	0.895

*Significant compared with time 0 and normal control (P<0.001) group.

In the same manner *C. anthelminticum*, *Cissus quadrangularis*, and *Woodfordia fruticosa Kurz* extracts demonstrated similar results with 500 mg/kg of oral administration, the blood glucose levels of rats decreased from 8.8 ± 0.11, 8.6 ± 0.10, and 9.0 ± 0.16 mmol/L, respectively, to 4.6 ± 0.11, 4.9 ± 0.07, and 4.8 ± 0.10 mmol/L after the second hour. This reduction continued by the fourth hour to 4.5 ± 0.14, 5.1 ± 0.07, and 4.7 ± 0.07 mmol/L, respectively. Finally, a slight increase was observed at 6 h (4.7 ± 0.17, 5.4 ± 0.16, and 5.1 ± 0.07 mmol/L). A significant difference in blood glucose levels was observed after the second, fourth, and sixth hours of oral administration with these extracts (P<0.001) when compared to that of the normal control group.

There was also a slight decrease in blood glucose levels by the *Sida acuta Burm F.* (P=0.002) and *Parthenium hysterophorus L.* (P=0.017) extracts after 2 and 4 h of oral administration, respectively.

Table 3. Hypoglycemicactivityofleaf extract by oral route in normal rats.

Group	Blood glucose levels (mmol/L)at different time points (h)				
	Time (mean ± SEM)				
	0h	2 h	4 h	6 h	*P*-value
Normal control (NaCl0.9%)	9.1 ± 0.12	8.9 ± 0.13	9.2 ± 0.17	9.5 ± 0.08	0.059
Glibenclamide 50mg/kg	9.2 ± 0.13	4.4 ± 0.06*	4.9 ± 0.16*	5.9 ± 0.12*	<0.001
Centratherum anthelminticum	8.8 ± 0.11	4.6 ± 0.11*	4.5 ± 0.14*	4.7 ± 0.17*	<0.001
Cissus quadrangularis	8.6 ± 0.10	4.9 ± 0.07*	5.1 ± 0.07*	5.4 ± 0.16*	<0.001
Woodfordia fruticosa Kurz	9.0 ± 0.16	4.8 ± 0.10*	4.7 ± 0.07*	5.1 ± 0.07*	<0.001
Madhuca indica	8.1 ± 0.09	8.3 ± 0.17	8.2 ± 0.17	8.4 ± 0.16	0.497
Vicoa indica Cass	8.9 ± 0.09	9.2 ± 0.06	8.8 ± 0.21	9.0 ± 0.20	0.354
Vanda tessellata (Roxb.)	8.7 ± 0.19	8.5 ± 0.19	8.5 ± 0.08	8.9 ± 0.08	0.285
Parthenium hysterophorus L.	9.5 ± 0.08	9.2 ± 0.09$	9.4 ± 0.06$	9.5 ± 0.12	0.017
Sida acuta Burm F.	8.6 ± 0.13	8.0 ± 0.11$	8.4 ± 0.14$	8.7 ± 0.07	0.002
Xanthium strumarium L.	9.2 ± 0.13	9.1 ± 0.21	9.4 ± 0.16	9.1 ± 0.13	0.747

*Significant compared with time 0 and normal control (P<0.001).
$Significant difference between treatment times in each group.

Intraperitoneal and Oral Glucose Tolerance Tests

Intraperitoneal and oral glucose tolerance tests were used to evaluate the effectiveness of the extracts with hypoglycemic potential as identified in the screening tests. When *C. anthelminticum*, *Cissus quadrangularis*, and *Woodfordia fruticosa Kurz* extracts were administered to overnight-fasted normal rats, no alteration in blood glucose levels was observed until 120 and 210 min after i.p. (250 mg/kg) and oral (500 mg/kg) administration, respectively.

Thereafter, at 30 min of i.p glucose injection, the level of blood glucose were greatly elevated in the (NaCl 0.9%) treated normal control rats (9.4 ± 0.23 mmol/L), followed by groups treated with *Woodfordia fruticosa Kurz* (7.5 ± 0.11 mmol/L),*Cissus quadrangularis*

(7.1 ± 0.13 mmol/L) and *C. anthelminticum* (6.4 ± 0.21 mmol/L), extracts(Fig. 1). Then at 60 min, the blood glucose levels had decreased in *C. anthelminticum* (4.8 ± 0.19 mmol/L), *Cissus quadrangularis* (5.9 ± 0.25 mmol/L), *Woodfordia fruticosa Kurz* (6.1 ± 0.22 mmol/L), as well as in the normal control (8.5 ± 0.15 mmol/L) groups. In the same manner reduction continued and was higher in the groups administered with all the three extracts (3.9 ± 0.22, 5.5 ± 0.19, and 5.1 ± 0.15 mmol/L, respectively) compared to that of normal control (6.7 ± 0.10 mmol/L) rat sup until 120 min. A significant difference in blood glucose levels was observed between the times for all the 3 extracts (P<0.001) respectively, when compared with the normal control (P<0.01) group.

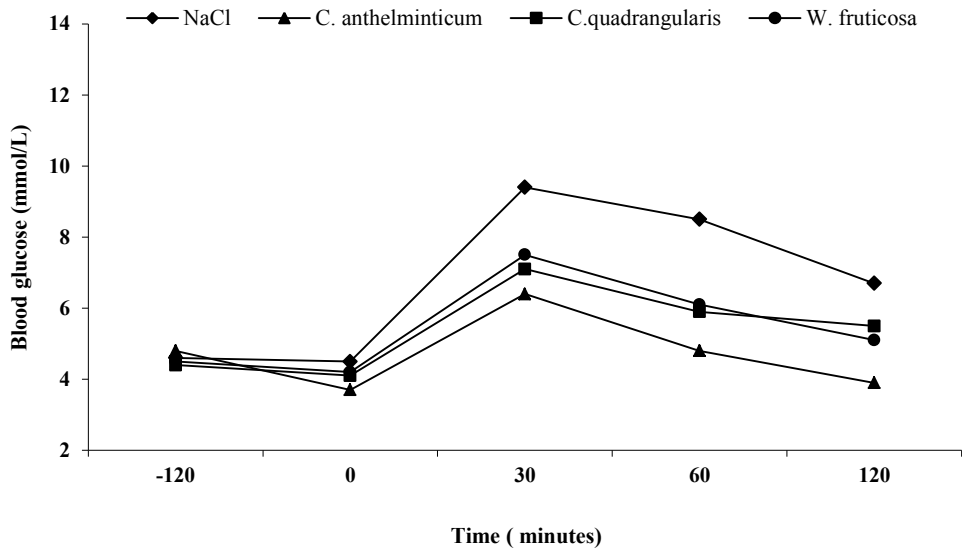

Figure 1. Intra-peritoneal glucose tolerance test on *C. anthelminticum*, *Cissus quadrangularis*, and *Woodfordia fruticosa Kurz* extracts in normal fasted rats with glucose-induced hyperglycemia. Data are mean ± SE. n = 6 for each group. (P<0.001) for the three extracts when compared with normal control (P<0.01) group.

In line with IPGTT, similar outcomes were obtained with Oral Glucose Tolerance Test. At 60 min, after glucose load the concentration of blood glucose were remarkably elevated to (11.3 ± 0.23 mmol/L) in the normal control group, followed by the groups treated with *Cissus quadrangularis* (9.0 ± 0.13 mmol/L), *Woodfordia fruticosa Kurz* (8.3 ± 0.13 mmol/L) and *C. anthelminticum* (7.5 ± 0.23 mmol/L) extracts (Fig. 2).Thereafter, at 120 min, the elevated blood glucose levels had significantly decreased in the groups administered with *C. anthelminticum* (4.3 ± 0.09 mmol/L), *Woodfordia fruticosa Kurz* (6.2 ± 0.12 mmol/L), and *Cissus quadrangularis* (7.2 ± 0.15 mmol/L) extracts, compared to that of the normal control (10.5 ± 0.15 mmol/L) group. In the same manner, the reduction of blood glucose levels was sustained for all the 3 extract groups (4.1 ± 0.12, 5.2 ± 0.10 and 6.5 ± 0.19 mmol/L, respectively) as well as for the control (7.8 ± 0.22 mmol/L) group by 180 min. A significant difference in blood glucose levels was observed between the times for all the 3 extracts (P<0.001) respectively, when compared with the normal control (P<0.01) group.

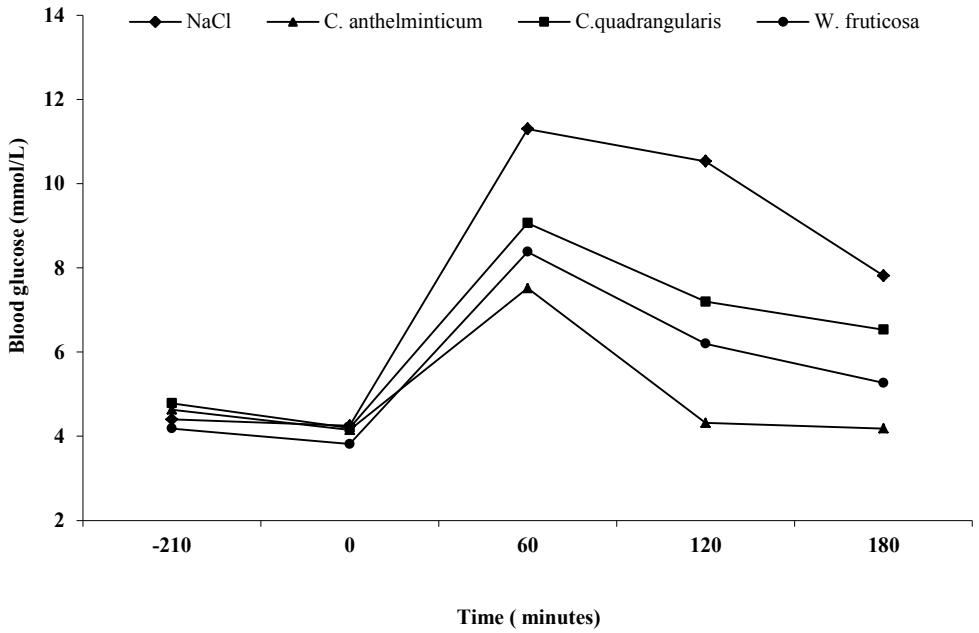

Figure 2. Oral glucose tolerance test on *C. anthelminticum*, *Cissus quadrangularis*, and *Woodfordia fruticosa Kurz* extracts in normal fasted rats with glucose-induced hyperglycemia. Data are mean ± SE. n = 6 for each group. (P<0.001) for the three extracts when compared with normal control (P<0.01) group.

The current study is a preliminary evaluation of the leaves from certain medicinal plants in the management of diabetes. The hypoglycemic activity of leaf extracts from 9 plants were screened and evaluated in normal rats. Of these nine plant extracts monitored, 5 (*C. anthelminticum*, *Cissus quadrangularis*, *Woodfordia fruticosa Kurz*, *Sida acuta Burm F.*, and *Parthenium hysterophorus L.*) showed direct effects in reducing postprandial hyperglycaemia in normoglycaemic rats, while four (*Vicoa indica*, *Vanda tessellata* (*Roxb.*), *Madhuca indica*, and *Xanthium strumarium*) did not, or else might have an indirect effect. Overall, *C. anthelminticum*, *Cissus quadrangularis*, and *Woodfordia fruticosa Kurz*leaves demonstrated the highest hypoglycemic activity. This finding was further verified by the glucose tolerance tests in normal fasted rats.

Firstly, our findings on *C. anthelminticum* leaf extracts supports previous data in which seed fraction exhibited anti-diabetic and anti-hyperlipidemic activity in diabetic rats without any toxicity.[6]The polyphenolic components from its seeds also exerted anti-hyperglycemic activity in maltose-loaded normal rats.[7]Apart from this, the plant is used extensively in Ayurveda for the treatment of various disorders. Experimental studies have also reported different pharmacological effects of the seed extracts.[13-14]In the same manner, extracts from different parts of *Cissus quadrangularis* and *Woodfordia fruticosa Kurz* also supports earlier findings[9-11] as well as with other biological activities.[15-20]Although acute toxicity studies point out the toxic nature of *Parthenium hysterophorus L.,* it possess slight hypoglycemic effects. Thus, *Parthenium hysterophorus L* should not be used in man until its toxicity has been more thoroughly determined. However, *Sida acuta Burm F.* also reported

minor hypoglycemic effects, thus did not support any previous reports, despite of other pharmacological responses.[21-22]

The different parts of *C. anthelminticum*, *Cissus quadrangularis*, and *Woodfordia fruticosa Kurz* plants are known to be a rich source of polyphenolic compounds,[7, 9, 23]therefore, our study plausibly propose that involvement of different active compounds in the extracts, including phenols might play an important role in the reduction of elevated blood glucose levels.[24]The availability of different medicinal plant parts in an appropriate time, the duration of these parts' lifespan, and the presence of the maximum amount of active compounds at that time are important issues in the pharmaceutical industry. Leaves remain available for a longer period on the plants compared to other plant parts, which is the reason we used leaves in our study. However, comparison of the distribution of the effective active compound levels between the different parts of these plants requires further study.

The aim in the management of diabetes mellitus is to attain blood glucose levels that are as close to normal as possible.[25] In addition, controlling postprandial hyperglycemia could also prevent the development of macro and microvascular complications associated with diabetes.[26]

Earlier studies on different plants, suggested that the administration of *Mucuna pruriens*,[27]*Cynara cardunculus*,[28] *Helicteres ixora*,[29]and *Tournefortia hartwegiana*[30]extracts facilitate diabetic patients in controlling of postprandial hyperglycemia, thus enhanced glucose tolerance activity in the patients.In line with this, our findings postulated that utilizing *C. anthelminticum*, *Cissus quadrangularis*, and *Woodfordia fruticosa Kurz* leaf extracts as dietary or food supplements may be advantageous for diabetic patients in the control of postprandial hyperglycemia.

There were some limitations in this study, and the main explanation for our results may varies in different solvents used during extraction due to the presence of active compounds, or the methods used to determine the hypoglycemic effect. Firstly, we focused on the leaves to test for hypoglycemic activity rather than other parts of the plants. Secondly, testing of the extracts was not done in a dose-dependent manner.

Conclusion

The current study on 9 plants, displayed that the leaf extracts of *C. anthelminticum*, *Cissus quadrangularis*, and *Woodfordia fruticosa Kurz* exhibited the highest hypoglycemic activity in normal glycemic rats. Therefore, further study to assess anti-diabetic activity from different parts of these plants is suggested in a dose-dependent manner along with new approaches.

Acknowledgments

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Antioxidant and Hypoglycemic Activities of Leaf Extracts of Three Popular *Terminalia* Species

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Abstract: This study aimed to ascertain the antioxidant and hypoglycemic activity of methanolic extracts of the leaves of *Terminalia arjuna*, *T. bellerica*, and *T. chebula*. Extracts were evaluated for total phenolic, flavonoid, and tannin content, and *in vitro* antioxidant potential with DPPH, ORAC, and FRAP assays. The extracts' hypoglycemic activities were evaluated by hypoglycemic screening and an oral glucose tolerance test (OGTT) in normal rats. The methanolic extracts of *T. chebula* leaves exhibited the highest quantity of total phenolic and flavonoid content, followed by those of *T. bellerica* and *T. arjuna*. *T. arjuna* contained more tannin than *T. bellerica* did, but less than that of *T. chebula*. The scavenging capacity of *T. chebula* for the antioxidant DPPH was the highest of the extracts tested, as it recorded the lowest IC_{50} value of all 3 extracts. Likewise, the results attributed the *T. chebula* extract with the highest oxygen radical absorption capacity (ORAC). In the FRAP assay, the extracts' ferric reducing antioxidant abilities were *T. arjuna* > *T. chebula* > *T. bellerica*. This correlates the potential of polyphenolic content enriched with antioxidant capabilities and substantiates the results of the hypoglycemic screening and OGTT, which determined that the *T. chebula* extract had a better hypoglycemic effect in normal and glucose-induced hyperglycemic rats (p <0.001) than that of *T. bellerica* and *T. arjuna*, respectively. The use of these *Terminalia* species as food supplements may help in reducing oxidative stress and related diabetic complications. The phytoconstituents responsible for the hypoglycemic activity need to be isolated to elucidate the relationship between the extracts' antioxidant capacity and their hypoglycemic effects.

Keywords: Polyphenols, Antioxidant activity, Hypoglycemic activity, Oral glucose tolerance test, *Terminalia chebula.*

Introduction

The use of natural products as antioxidants in the management of diabetes mellitus has gained importance throughout the world. There has been increasing research focused on natural foods and medicinal plants and their phytoconstituents due to their well-known abilities to scavenge free radicals, *i.e.,* antioxidative ability[1-3]. The interest in natural antioxidants has burgeoned since evidence of their potential interference in the production of reactive oxygen species (ROS) was uncovered, these ROS plays an important role in the progression of a great number of pathological disturbances such as inflammation, atherosclerosis, stroke, heart disease, diabetes mellitus, multiple sclerosis, cancer, Parkinson's disease, Alzheimer's disease, *etc*[4-5].

The world health organization has also recommended and encouraged the use of natural products for the management of diabetes and in oxidative stress conditions, especially in countries where access to conventional treatment of diabetes is inadequate. There is an increased demand for natural products with antidiabetic activity due to the side effects associated with the use of insulin and oral hypoglycemic agents[6].

Natural antioxidants such as flavonoids and polyphenols are believed to possess antioxidant properties due to their reducing and chelating capabilities. Flavonoids and polyphenols are secondary plant metabolites that are widely distributed in fruits, leaves, bark, and other parts in plants with free radical scavenging abilities[7]. The genus *Terminalia* (fam. Combretaceae), comprising 250 species, is distributed across tropical countries worldwide, and the Indian traditional system of medicine has documented several books and literatures on the medicinal values of many of its species, of which *Terminalia bellerica*, *T. chebula* and *T. arjuna* are prime examples[8-9]. It has been reported that these species are rich in flavonoids and polyphenols. In "Ayurveda," a herbal formulation combining the dried fruits of *T. chebula*, *T. bellerica* and *Emblica officinalis* by the name of "Triphala" has been used as a food and dietary supplement to derive several health benefits such as laxation, detoxification, liver protection, anti-aging, and as a rejuvenator of the body[10-11]. The combination has also been found to have antidiabetic and cholesterol-lowering activities[12-13]. The fruit extracts of *T. chebula* and *T. bellerica* have been shown to contain antioxidants[14], and *T. arjuna* bark extracts have been reported to possess cardioprotective, antioxidant, and antimutagenic abilities[15-17]. Despite reports of the antioxidant and antidiabetic activities of the fruit and bark of *Terminalia* species, which are a rich source of flavonoids, tannins, and many phenolic derivatives, concrete evidence supporting the relationship with regard to the leaves of the genus is lacking.

Therefore, the present study was planned to investigate methanolic leaf extracts of the 3 popular species to determine their total phenolic (TPC), total flavonoid (TFC), and total tannin content (TTC), as well as their 1,1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging activity, oxygen radical antioxidant capacity (ORAC), and ferric reducing antioxidant power (FRAP) in correlation to their hypoglycemic activity.

Experimental

Dried powdered leaves of *T. arjuna*, *T. bellerica,* and *T. chebula* were obtained from Amritum Bio-Botanica Herbs Research Laboratory Pvt. Ltd, Jogli, India. These samples were successively defatted with hexane, then chloroform, and finally with methanol using a

Soxhlet apparatus. The resulting methanol extracts were evaporated under reduced pressure at 40°C using a rotary evaporator to derive a crude methanol (MeOH) extract that was further lyophilized and stored at -20°C prior to use.

Determination of Total Phenolic and Flavonoid Content

The TPC and TFC of the methanolic extracts were determined by the Folin-Ciocalteu and Dowd methods as adapted by Lamien-Meda *et al*[18]. Results are expressed as mg Gallic Acid equivalents (GAE) and mg Quercetin (Q) equivalents.

Determination of Total Tannin Content

The TTC in the methanolic extracts was estimated with the method by Price and Butler[19] with some modifications. A 3.0 mL volume of the sample, 3.0 mL of vanillin (4%) in methanol and 1.5 mL con. HCl were mixed and were incubated in the dark for 10 min. Subsequently, the TTC content of the samples were analyzed with a UV-Vis. spectrophotometer at 500 nm. Results are expressed as mg Catechin (C) equivalents.

In vitro antioxidant study

DPPH Radical Scavenging Activity

The scavenging activity of all the 3 methanolic extracts on DPPH (1,1-diphenyl-2-picrylhydrazyl) was determined using the method described by Choi *et al* [20] with slight modifications. This method is based on the reduction of purple DPPH to a yellow colored diphenylpicrylhydrazine. Changes in color were measured at 518 nm. All the extracts were tested at final concentrations ranging 600–10 µg/mL in ethanol. One milliliter of 0.3 mM DPPH ethanol solution was added to 2.5 mL of sample solution in different concentrations to produce the test solutions, while 1 mL of ethanol was added to 2.5 mL of sample to produce the blank solutions. The negative control consisted of 1 mL of DPPH solution plus 2.5 mL of ethanol. The solutions were allowed to react at room temperature for 30 min in the dark. The absorbance values were measured at 518 nm and converted into percentage antioxidant activity using the following equation:

$$\% \text{ Inhibition} = [(AB - AA)/AB] \times 100,$$

where AB: absorption of blank sample; AA: absorption of tested samples. The half maximal inhibitory concentration (IC_{50}) and the kinetics of DPPH scavenging activity were determined. Ascorbic acid and butylated hydroxytoluene (BHT) were used as positive controls in this assay.

ORAC Antioxidant Activity Assay

The oxygen radical absorbance capacity (ORAC) assay was carried out based on the procedure described by Cao G *et al*[21] with slight modifications. Briefly, 175 µL of the sample/blank were dissolved with PBS at concentrations of 160 µg/mL, pH 7.4. Serial dilutions of the standard Trolox were prepared from 75 mM. The assay was performed in 96-well black microplates, to which 25 µL each of samples (extracts), standard (Trolox), blank (solvent/PBS), or positive control (quercetin) were added. Subsequently, 150 µL of fluorescent sodium salt solution was added, and the plate was incubated for 45 minutes at 37°C. 2,20-azobis (2-amidinopropane) dihydrochloride (AAPH) solution (25 µL) was added to make up a total volume of 200 µL/well. Fluorescence was recorded at 37°C until it

reached 0 (excitation at 485 nm, emission at 535 nm) using a fluorescence spectrophotometer (Perkin-Elmer LS 55) equipped with an automatic thermostatic autocell-holder. Data were collected every 2 min for 2 h and were analyzed by calculating the differences of areas under the fluorescein decay curve (AUC) between the blank and the sample. Values are expressed as Trolox equivalents.

FRAP Assay

The FRAP (ferric reducing/antioxidant power) assay was modified from the method used by Benzie and Strain[22]. The stock solutions included 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl and 20 mM $FeCl_3 \cdot 6H_2O$ solution. A fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ, and 2.5 mL $FeCl_3 \cdot 6H_2O$. The temperature of the solution was raised to 37°C before use. Extracts (10 µL) were allowed to react with 190 µL of FRAP solution for 30 min in the dark. Colorimetric readings of the product, *i.e.*, the ferrous-TPTZ complex, were taken at 593 nm for 10 min and a steady state was reached within 5 min for the different test substance concentrations. The EC_1 value was calculated from the regression curve as the concentration of antioxidant (lM) giving an absorbance reading equivalent to that obtained with a 1 mM Fe(II) solution. The standard curve was linear between 200 and 1000 µM $FeSO_4$. Results are expressed as µM Fe (II)/g dry mass and compared with those of ascorbic acid and BHT.

***In Vivo* Study**

Animals and Treatment

Male Sprague-Dawley rats weighing 200–250 g were procured from the Animal Care Unit of the University Malaya Medical Centre (UMMC) in Kuala Lumpur, Malaysia and maintained under pathogen-free conditions in the animal housing unit in a temperature-(23±2°C) and light-controlled (12 h light/dark cycle) room with 35–60% humidity. Animals were provided with carbohydrate-enriched rodent chow and water *ad libitum*. The Animal Ethics Committee from the UMMC Faculty of Medicine approved the study under Approval No. FAR/10/11/2008/AA(R).

Acute Oral Toxicity

The acute oral toxicity test of the 3 plants' methanolic extracts was planned according to the Organization for Economic Co-operation and Development (OECD) guidelines. Healthy male Sprague-Dawley rats weighing 150–200 g were used for this study. After an overnight fast, the rats were divided into 6 groups (n = 9–10) and orally fed with the methanolic leaf extracts. Three groups were fed with doses of 50 mg/kg body weight and the other 3 groups were fed doses of 2500 mg/kg body weight. The rats were observed continuously for 24 h for any behavioral, neurological, and autonomic profiles, and after 72 h for any lethality. According to this toxicity study, 1/10 of the maximum dose administered was used for the hypoglycemic screening study.

Determination of Postprandial Blood Glucose Levels in Normal Rats

Animals were divided into 5 groups of 8–9 in a group. One group was used as a positive control and received glibenclamide (5 mg/kg) orally; the negative control group received NaCl (0.9%, dose 2 mL/200 g), while the other 3 groups received methanolic leaf extracts (250 mg/kg). The lyophilized powder of all leaf extracts was reconstituted with filtered water and administered orally using a gavage (2 mL/200 g body weight). Blood samples were obtained by amputation of the tail tip in non-fasting conditions after 2, 4 and 6 h of treatment. Glycemia was determined using the glucose oxidase-peroxidase enzymatic method (Accu-Check Performa, Roche Diagnostic Germany) through electronic glucometer.

An extract was deemed to have a hypoglycemic effect if the blood glucose levels of the rats decreased significantly compared to those of the normal control group[23].

Glucose Challenge (Glucose Tolerance Test)

An oral glucose tolerance test (OGTT) was used to evaluate the effectiveness of the leaf extracts with hypoglycemic potential on glucose induced hyperglycemic rats, which was derived from prior screening tests and from which the positive results formed the basis of this hypoglycemic screening. In rats fasted overnight, extracts (250 mg/kg) were fed in doses of 2.0 mL/200 g body weight and the control group was given NaCl (0.9%). Concentration of glucose level in blood was measured before and 120 min after extracts administration. After this time point, glucose (3 g/kg) was introduced orally, then glucose concentration in blood was measured at 30, 60 and 120 min post-challenge[23].

Statistical Analysis

The data are expressed as the mean ±SD of 3 measurements. Statistical analysis was performed using one-way and two-way repeated measures ANOVA and comparison of the data was done by Tukey's test with all pair-wise multiple comparison procedures. $p < 0.001$ was considered statistically significant.

Results and Discussion

In this study, we evaluated the total phenolic, total flavonoid, and total tannin content in the methanolic extract of *T. chebula*, *T. bellerica* and *T. arjuna* leaves, followed by evaluation of the extracts' antioxidant activities and *in vivo* hypoglycemic screening in normal rats.

Total Phenolic, Flavonoid and Tannin Content

There has been an increased desire in consumers for functional foods with antioxidant capabilities. The fruit and leafy parts of plants are considered rich in polyphenols and flavonoids, which contributes to their antioxidant capacity[24]. The antioxidant property of phenolic compounds is attributed to their ability to absorb and neutralize free radicals.

The results in Table 1 demonstrate that the methanolic extract of *T. chebula* leaves contained the highest TPC, followed by those of *T. bellerica* and *T. arjuna* (266.16, 259.28, and 147.23 mg GAE/g extract, respectively), likewise, the TFC were 29.23, 16.15, and 8.19 mg Q/g extract, respectively. *T. chebula* possessed the highest TTC, followed by *T. arjuna* and *T. bellerica* (8.36, 4.68 and 6.31 mg C/g extract, respectively). *T. chebula* fruits have been found to contain higher amounts of total phenolics, total flavonoids and total tannin, and are reported to contain gallic acid, chebulic acid, 1,6-di-*O*-galloyl-*β*-*D*-glucose, punicalagin,3,4,6-tri-*O*-galloyl-*β*-*D*-glucose, casuarinin, chebulanin, chebulagic acid, chebulinic acid and 1,2,3,4,6-penta-*O*-galloyl-*β*-*D*-glucosein, which might be responsible for its high antioxidant activity[25].

Table 1. Total phenolic, flavonoid, and tannin content in methanolic extracts of *Terminalia chebula, T. bellerica*, and *T. arjuna* leaves.

No.	Plants	Total Phenolic Content[a] (mg GAE/g extract)	Total Flavonoid Content[a] (mg Quercetin/g extract)	Total Tannin Content[a] (mg Catechin/g extract)
1	*T. chebula*	266.16 ± 7.81	29.23 ± 3.81	8.36 ± 0.37
2	*T. bellerica*	259.28 ± 6.42	16.15 ± 2.42	4.68 ± 1.31
3	*T. arjuna*	147.23 ± 2.34	8.19 ± 1.41	6.31 ± 0.17

[a]*Values expressed are mean ± SD of triplicate measurements.*

In Vitro Antioxidant Activities

The antioxidant activities of the *T. chebula*, *T. bellerica* and *T. arjuna* leaf extracts were determined with DPPH, ORAC and FRAP assays. These assays were measured in triplicate at different concentrations to determine the extracts' IC_{50} values. The results are listed in Table 2.

DPPH Radical Scavenging Activity

The DPPH radical is used commonly and extensively to determine the *in vitro* antioxidative activity of antioxidant compounds. When treated with substances or samples that are hydrogen atom donors, the DPPH radical is converted into a stable DPPH radical, indicated by a color change from purple to yellow (Kriengsak Thaipong, 2006). DPPH radical reduction is analyzed by measuring the absorbance of samples at a 518 nm wavelength. The DPPH scavenging capacity of *T. chebula* (11.6 µg/mL) was the highest of the 3 extracts tested, as it recorded the lowest IC_{50} value of all the extracts, while *T. bellerica* (16.4 µg/mL) demonstrated better scavenging activity than *T. arjuna* (21.8 µg/mL) did. Among the positive controls, ascorbic acid (1.6 µg/mL) was found to have better DPPH radical scavenging ability than BHT (1.7 µg/mL). Flavonoids are well-known antioxidant constituents of plants and possess a broad spectrum of chemical and biological activities, including radical-scavenging or chelating activities[26].

ORAC Assay

All the extracts demonstrated good oxygen radical absorption capacity; IC_{50} values are expressed in Trolox (µM/mL) equivalent (TE) concentrations as listed in Table 2. *T. chebula* exhibited lower IC_{50} values (18.23 µM TE/mL) than the other 2 species did. *T. arjuna* had an IC_{50} value of 42.31 µM TE/mL, whereas that of *T. bellerica* was 29.17 µM TE/mL. The positive control (Quercetin) had the lowest IC_{50} value (12.16 µM TE/mL). Therefore, the results attribute *T. chebula* extracts with the highest oxygen radical absorption capacity. The ORAC assay is based upon the inhibition of the peroxyl-radical-induced oxidation initiated by thermal decomposition of azo-compounds such as [2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH)].

Reactive oxygen species (ROS) are hazardous to cellular structures and functional molecules (*i.e* DNA, proteins, lipids) as they act as strong oxidizing mediators or free radicals. Biological antioxidants are able to dispose of ROS; as they are found to be safe and effective in eliminating all of the free radicals, oxygen ions and peroxides that can do damage to the body[7]. In this manner, the ORAC assay uses a biological relevant radical source and it combines both inhibition time and degree of inhibition into one quantity[21].

FRAP Assay

The principle of a FRAP assay involves the reduction of ferric ions to ferrous ions by plant extracts due to the presence of reducing substances in the extracts[27]. The extracts' ability to reduce ferric ions was determined using the FRAP assay developed by Benzie and Strain[10]. Electron-donating antioxidant compounds are capable of reducing the ferric-TPTZ (Fe(III)-TPTZ) complex to a blue ferrous-TPTZ (Fe(II)-TPTZ) complex that exhibits strong absorbance at 593 nm. Evaluation of the extracts' ferric reducing antioxidant ability determined that the EC_1 value of *T. arjuna* (232 µg/mL) was lower than that of *T. chebula* (243 µg/mL) and *T. bellerica* (265 µg/mL). The lower the EC_1 value, the higher the ferric reducing antioxidant abilities of the extract. The BHT (3.2 µg/mL) standard had greater ferric ion reducing capability than ascorbic acid (3.7 µg/mL) did. The reducing capacity of *T. arjuna* leaf extract may have been due to a large number of polyphenolic compounds with electron-donating hydroxyl groups.

Table 2. Antioxidant activities of *Terminalia chebula*, *T. bellerica* and *T. arjuna* methanolic leaf extracts in DPPH, ORAC and FRAP assays.

No.	Samples	[a]DPPH IC_{50}, µg/mL	[a] ORAC IC_{50}, µM/mL	[a]FRAP EC_1, µg/mL
1	*T. chebula*	11.6±0.43	18.23±0.9	243±5.8
2	*T. bellerica*	16.4±0.55	29.17±1.8	265±6.7
3	*T. arjuna*	21.8±0.37	42.31±2.7	232±8.3
4	Ascorbic acid	1.6±0.14	Not applicable	3.7±0.8
5	BHT	1.7±0.07	Not applicable	3.2±0.5
6	Quercetin	Not applicable	12.16±1.3	Not applicable

ORAC: Equivalent conc. Trolox (20 µg/mL) (µM) [a]DPPH, [a]ORAC and [a]FRAP assays demonstrating low values indicate high antioxidant activity. Standard deviation (SD) values of a minimum of 3 replicates.

In Vivo Study

Acute Oral Toxicity Studies

The leaf extracts of the 3 selected species were found to be non-toxic in rats, as neither mortality nor any considerable symptoms of toxicity were observed after oral administration of extracts at 2 dose levels (50 and 2500 mg/kg body weight) over a 72-hr period.

Hypoglycemic Activity in Methanolic Leaf Extracts by Oral Route

There was a reduction in blood glucose levels at 2 and 4 hr, with a slight increase at 6 h after oral administration of *T. chebula*, *T. bellerica* and *T. arjuna* leaf extracts. After glibenclamide administration, blood glucose levels were significantly reduced at 2, 4, as well as 6 h, but blood glucose levels did not change significantly within the different time intervals after oral administration of the negative control (0.9% NaCl). The results are listed in Table 3. There was a significant difference in blood glucose levels at 2, 4 and 6 h after oral administration of glibenclamide, *T. chebula, T. bellerica*, and *T. arjuna* extracts (p <0.001) as compared to levels at 0 h and in the negative control (NaCl) group.

The control of postprandial hyperglycemia is one of the beneficial therapies for management of type 2 diabetes mellitus[28], along with nutrition and oral hypoglycemic and insulin therapies[29]. In order to determine a scientific basis for the utilization of *T. chebula*, *T. bellerica* and *T. arjuna* leaves in the treatment of diabetes, we decided to evaluate their extracts' hypoglycemic effect in normal rats. Earlier reports revealed that *T. chebula* and *T. bellerica* fruits and *T. arjuna* bark possessed antidiabetic effects when studied in STZ-induced diabetic animals, and suggested that most diabetic complications were mediated through oxidative stress[30]. Production of ROS and its related oxidative stress was reported as the root cause for the development of insulin resistance, β-cell dysfunction, impaired glucose tolerance and type 2 diabetes mellitus[31]. The present study demonstrated that *T. chebula* leaf extract was more effective at decreasing blood glucose in normal rats, followed by *T. bellerica* and *T. arjuna* when compared to the negative control. This may be due to their flavonoid and phenolic contents, which are known to be involved in the healing process of free radical-mediated diseases, including diabetes[32].

Table 3. Hypoglycemic activity of methanolic leaf extracts by oral route in normal rats.

Group	Alteration in blood glucose levels (mmol/L) before and after administration of test samples (mean ± SD)				
	0 h	2 h	4 h	6 h	*p*-value
NaCl control	7.5±0.32	7.1±0.23	6.6±0.37	6.7±0.28	0.059
Glibenclamide	7.2±0.41	4.6±0.33*	4.5±0.24*	4.1±0.45*	<0.001
T. chebula	6.9±0.37	5.4±0.26*	4.9±0.52*	5.1±0.31*	<0.001
T. bellerica	6.6±0.29	4.9±0.17*	5.1±0.37*	5.4±0.46*	<0.001
T. arjuna	7.0±0.36	5.8±0.24*	5.2±0.38*	5.7±0.29*	<0.001

**Comparison with time 0 and negative control (NaCl) indicates significance (p <0.001).*

Oral Glucose Tolerance Test

An oral glucose tolerance test was used to confirm the result of the hypoglycemic effects. The results depicted in Fig 1. indicate that there was no major alteration in blood glucose levels until 120 min following administration of extracts and NaCl in overnight-fasted normal rats. However, after 30 min of glucose load, blood glucose levels were considerably elevated in all treated groups up until 60 min, followed by a fall in glucose concentration until 120 min. Hence, there was a significant difference in blood glucose levels throughout the observation period for all 3 leaf extracts ($p < 0.001$ when compared with the normal control group). This suggests that methanolic leaf extracts of Terminalia species have the ability to improve glucose tolerance activity in normal rats. This observation indicates that the extracts may interfere with the intestinal glucose absorption in the gut by various mechanism, this may be postulated that the positive results of these extracts might stimulates glycogenesis in the liver, which is enhanced by feeding and is hypothesized that the glucose tolerance activity induced by the extracts could be due to a mixture of active compounds that reduce the concentration of glucose and improve glucose tolerance activity[33-34].

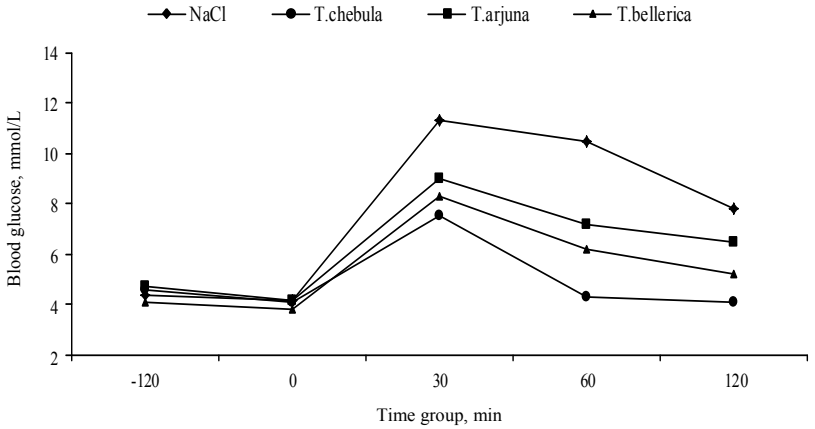

Figure 1. In normal fasted rats blood glucose levels, significantly changed at different time intervals after oral administration of extracts and glucose load ($p < 0.001$).

Conclusion

The present study indicates that the leaf extracts of the selected *Terminalia* species possess antioxidant and hypoglycemic activities, which is probably due to their phenolic groups, and

brings new hope to research on the management of type 2 diabetic conditions. The use of these species as food supplements may aid in reduction of oxidative stress and related diabetic complications. However, the mechanism of action by which these species exert their action needs to be established by a thorough phytochemical investigation to identify the constituents responsible for the antioxidant and hypoglycemic activity. Furthermore, long-term studies on type 2 diabetic models must be carried out to describe the exact mechanism of action at the cellular level.

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Anti-diabetic effects of *Centratherum anthelminticum* seeds methanolic fraction on pancreatic cells, β-TC6 and its alleviating role in type 2 diabetic rats

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ABSTRACT

Ethnopharmacological relevance: Seeds of *Centratherum anthelminticum* (Asteraceae) have been popularly used in Ayurvedic medicine to treat diabetes and skin disorders. Folk medicine from Rayalaseema (Andhra Pradesh, India) reported wide spread usage in diabetes.

Aim of the study: To investigate the hypoglycemic properties and mechanism of the methanolic fraction of *C. anthelminticum* seeds (CAMFs) on mouse *β*-TC6 pancreatic cell line and streptozotocin (STZ)-induced diabetic rat models.

Materials and Methods: We investigated the crude methanolic fraction of *C. anthelminticum* seeds (CAMFs) on β-TC6 cell line and confirmed its effects on type 1 and type 2 diabetic rats to understand its mechanism in managing diabetes mellitus. CAMFs were initially tested on β-TC6 cells for cytotoxicity, 2-NBDG glucose uptake, insulin secretion and glucose transporter (GLUT-1, 2 and 4) protein expression. Furthermore, streptozotocin (STZ)-induced type 1 diabetic and STZ-nicotinamide-induced type 2 diabetic rats were intraperitoneally (i.p) injected or administered orally with CAMFs daily for 28 days. The effect of CAMFs on blood glucose and insulin levels was subsequently evaluated.

Results: In cell line studies, CAMFs showed non-cytotoxic effect on β-TC6 cell proliferation compared to untreated control cells at 50 μg/ml. CAMFs increased glucose uptake and insulin secretion dose-dependently by up-regulating GLUT-2 and GLUT-4 expression in these cells. Further *in vivo* studies on streptozotocin induced diabetic rat models revealed that CAMFs significantly reduced hyperglycemia by augmenting insulin secretion in type 2 diabetic rats. However, CAMFs displayed less significant effects on type 1 diabetic rats.

Conclusions: CAMFs demonstrated anti-diabetic potential on β-TC6 cells and type 2 diabetic rat model, plausibly through enhancing glucose uptake and insulin secretion.

1. Introduction

Diabetes mellitus (DM) is a complex metabolic disorder characterized by high blood glucose level due to defects in insulin secretion, insulin action or both. It affects the metabolism of carbohydrates, fats, proteins and electrolytes in the body. Type I diabetes (DM1) or insulin-dependent diabetes mellitus (IDDM) occurs due to immunological destruction of pancreatic β cells and consequent insulin deficiency. Type II diabetes (DM2) or non-insulin-dependent diabetes mellitus (NIDDM) is characterized by impaired insulin secretion or insulin resistance. DM2 is usually associated with obesity and hereditary disposition and it is the most common form of diabetes, affecting 90–95% of cases (Tamrakara et al., 2011). Resistance to insulin impairs the sensitivity of the main target organs (muscle, liver and adipose tissues) to the hormone, which increases circulating FFA concentrations, inhibits muscle cell's glucose uptake and enhances glucose

Abbreviations: 2-NBDG, 2-[*N*-(7-nitrobenz-2-oxa-1,3-diaxol-4-yl)amino]-2-deoxyglucose; ATCC, American Type Culture Collection; bw, body weight; CA, *Centratherum anthelminticum*; CAMFs, methanolic fraction of *C. anthelminticum* seeds; CI, cell index; DM1, type 1 diabetes; DM2, type 2 diabetes; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; GC–MS, gas chromatography–mass spectrometry; GLUT, glucose transporter; FFA, free fatty acid; HCS, high content screening; HRP, horseradish peroxidase; IC_{50}, 50% inhibitory concentration; IDDM, insulin-dependent diabetes mellitus; IP, intraperitoneal; LCMS-MS, liquid chromatography–tandem mass spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NIDDM, non-insulin-dependent diabetes mellitus; OECD, Organization for Economic Co-operation and Development; OGTT, oral glucose tolerance test; PBS, phosphate-buffered saline; PBS-T, PBS-Tween 20; PPARs, peroxisome proliferator-activated receptors; PVDF, polyvinylidene fluoride; r^2, coefficient of determination; RTCA, Real-Time Cellular Analysis; SD, standard deviation; SDS, sodium dodecyl sulfate; STZ, streptozotocin; TMB, 3,3',5,5'-tetramethylbenzidine

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production by the liver. These conditions are considered as DM2 hallmarks (Reaven, 1988; Defronzo, 2009). Currently, the range of medication is limited. None of the available options is able to vigorously enhance insulin secretion and sensitivity simultaneously (Cohen and Horton, 2007). Thus, it is obvious that there is an urgent need for more effective therapeutic agents.

The plant kingdom holds great potential to meet this need. However, scientific testing and validation of the efficacy of most medicinal plants in alleviating DM1 and DM2 is rare. Thus, we have limited knowledge of their safety and efficacy, as most of the data is based on information obtained from traditional medicinal plant practitioners (Smirin et al., 2010). Folk medicine from Rayalaseema reports 26 plants for the management of diabetes. One such plant is *Centratherum anthelminticum* (L.) Kuntze, which belongs to the family Asteraceae (Nagaraju and Rao, 1990). Seed of this plant is being used by Indian traditional healers to treat diabetic conditions. The trade name of *C. anthelminticum* is wild cumin and it is also known as Kalizeeri in Hindi. *C. anthelminticum* is one of the major contents for the Ayurvedic formulation, e.g., Kayakalp, a preparation used for the whole-body rejuvenation. Pharmacological investigations of *C. anthelminticum* (CA) seeds demonstrated various biological activities, including anti-cancer, anti-diabetes and anti-inflammation (Ani and Naidu, 2008; Fatima et al., 2010; Arya et al., 2012). However, little is known on the anti-diabetic effect of the defatted crude methanolic fraction of *C. anthelminticum* seeds (CAMFs).

The present study was undertaken to scientifically investigate anti-diabetic potential of CAMFs using *in vitro* mouse pancreatic β-TC6 cells and *in vivo* diabetic rat models. We first studied cytotoxicity and cell proliferation assay on β-TC6 cells. Furthermore, we investigated CAMFs effects on *in vitro* glucose uptake in β-TC6 cells using 2-[*N*-(7-nitrobenz-2-oxa-1,3-diaxol-4-yl)amino]-2-deoxyglucose (2-NBDG) and insulin secretion. Next, we performed Western blotting to evaluate glucose transporter protein (GLUT-1, 2 and 4) expression after CAMFs treatment. Subsequently, we investigated the effect of CAMFs on glucose levels and insulin secretions in streptozotocin (STZ)-induced type 1 and STZ-nicotinamide-induced type 2 diabetic rat models.

2. Material and methods

2.1. Preparation of CAMFs

2.1.1. Collection of plant material

Dried seeds of *C. anthelminticum* were obtained from the medicinal plant cultivation zone of Amritum Bio-Botanica Herbs Research Laboratory Pvt. Ltd. (Madhya Pradesh, India). The dried seeds were authenticated by the company's quality control department. Voucher specimens of the seeds (CA-9) were deposited with the company and in the Department of Pharmacology, Faculty of Medicine, University of Malaya (UM).

2.1.2. Extraction and fractionation

The seeds of *C. anthelminticum* (2 kg) were pounded using grinder and extracted with 100% *n*-hexane using a Soxhlet extractor for 24 h. The solvents were then removed under reduced pressure at a maximal temperature of 40 °C to get viscous greenish oil. Then, the obtained residue was further fractionated by 100% chloroform, to get semisolid fraction. Finally, the defatted residue was again fractionated with absolute methanol to get defatted crude methanolic fraction (CAMFs). The final fraction was then freeze-dried and stored at −20 °C until further use.

2.1.3. Phytochemical analysis of CAMFs by LCMS-MS

Analysis of the major compounds in CAMFs was done using liquid chromatography–tandem mass spectrometry (LCMS-MS). A triple quadrupole mass spectrometer equipped with a turbo ion spray source (AB SciexQTrap 5500, Ontario, Canada) was used to obtain the MS/MS data in negative ion mode. 0.1% formic acid in water and in acetonitrile (ACN) were used as mobile phase by eluting at a flow rate of 0.4 mL/min with an injection volume of 20 μL. Separation of the compounds was performed using a Luna 3-μm RP C18 column (100 mm × 2.00 mm; Phenomenex). The turbo ion source settings were as follows: capillary voltage, −4000 V; dry gas flow (N_2), 9 L/min; nebulizer pressure, 35 psi; and capillary temperature, 365 °C. A full scan of the mass spectra was recorded from *m*/*z* 50 to *m*/*z* 1000. The acquisition data was processed with Analyst Software version 1.5.1. Compounds were characterized based on their UV spectra and MS^2 and MS^3 fragmentations spectra data by correlation with the previous reports.

2.1.4. Phytochemical analysis of CAMFs by GC–MS

The analysis of CAMFs by GC/MS was carried out using a Shimadzu GC-17A network GC system coupled to a mass detector with a SGE BPX35 MS (25 m × 0.22 mm × 0.25 μm) capillary column. Helium gas was used as the carrier gas at a flow rate of 1 mL/min. The injection volume was 1 μL and the samples were injected in split mode as 10:1. The column temperature was initially set at 100 °C for 3 min, raised to 250 °C at the rate of 10 °C/min, and maintained at this temperature for 3 min. The injector and detector temperatures were set at 250 °C and 280 °C, respectively. The ionization voltage was 70 eV with the mass scan operating in the range of 40–450 amu. Identification of the major compounds was based on the comparison of mass spectra and retention times with those of standards from the database of National Institute Standards and Technology (NIST21 and NIST Wiley).

2.2. In vitro assays

2.2.1. Cell culture

Mouse β-TC6 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in 15% fetal bovine serum (FBS) in Dulbecco's Modified Eagle Medium (DMEM). Cultures were maintained at 37 °C in 5% CO_2 in a humidified incubator. The growth medium was changed every 3 days.

2.2.2. Cellular viability

The β-TC6 cells were used to determine the effect of CAMFs on cell growth using the MTT assay. The MTT assay was performed as described by Mosmann (1983) with slight modification. Briefly, cells were seeded at a density of 1.5×10^4 cells/mL in a 96-well plate and incubated for 24 h at 37 °C in 5% CO_2. The next day, cells were treated with CAMFs and incubated for another 24 h, following which 2 mg/mL MTT solution was added for 1 h. Absorbance at 570 nm was measured and recorded using a Plate Chameleon V microplate reader (Hidex, Turku, Finland). Results are expressed as a percentage of control cells demonstrating percentage cell viability after 24-h exposure to CAMFs. The potency of cell growth was expressed. Viability was defined as the ratio (expressed as a percentage) of the absorbance of treated cells to that of the untreated cells.

2.2.3. Real-time cell proliferation

In vitro cell proliferation was assessed using an xCELLigence Real-Time Cellular Analysis (RTCA) system (Roche, Manheim, Germany). Briefly, background measurements were obtained after

adding 50 μL of the appropriate medium to the wells of the 16X E-plate, and 1.5×10^4 cells were added to each well. Cell attachment and proliferation were monitored every 5 min using the RTCA system. Approximately 17 h after seeding, when the cells were in the log growth phase, the cells were treated with 100 μL of CAMFs in concentrations of 3.125, 6.25, 12.5, 25, 50, and 100 μg/mL dissolved in cell culture media, and continuously monitored for up to 72 h. The cells were also treated with medium alone, which served as the vehicle control. Cell sensor impedance was expressed as an arbitrary unit called the cell index (CI).

2.2.4. 2-NBDG glucose uptake

The uptake of the fluorescent hexose 2-NBDG, a glucose analog, was assayed as described by Loaiza et al. (2003) with modifications. Briefly, 1.5×10^4 cells were seeded onto a 96-well plate and allowed to attach, spread, and proliferate to near confluence at 37 °C in 5% CO_2. The medium was then removed and the cells were washed with phosphate-buffered saline (PBS) twice. The PBS was then replaced with 2.5 mM glucose in basal medium comprising DMEM without glucose or pyruvate supplemented with L-glutamine and 15% (v/v) FBS (final serum glucose concentration of approximately 0.25 mM). Conditioning of the cells proceeded at 37 °C in 5% CO_2 for 60 min. The conditioning medium was then removed and replaced with 10 mM 2-NBDG (Invitrogen, Carlsbad, CA, USA) in basal medium in the presence or absence of CAMFs. The cells were then incubated at 37 °C in 5% CO_2 for 30 min to permit endocytosis of the 2-NBDG, with the selected concentration being the minimum concentration capable of producing an adequate signal-to-noise ratio. The 2-NBDG was then removed, the cells washed with PBS, and stained with the nucleic dye Hoechst 33342 for another 30 min. The cells were then observed for intra-cellular fluorescence at *Ex*/*Em* = 350 nm/461 nm and *Ex*/*Em* = 475 nm/550 nm for Hoechst 33342 and 2-NBDG, respectively. Plates were evaluated using the ArrayScan High Content Screening (HCS) system (Cellomics Inc., Pittsburgh, PA, USA) and analyzed with Target Activation BioApplication software (Cellomics Inc.).

2.2.5. In vitro insulin secretion

The β-TC6 cells (1.5×10^5 cells/mL) were seeded onto a 24-well plate and allowed to attach, spread, and proliferate at 37 °C in 5% CO_2. After reaching 80–90% confluence, the cells were washed with glucose-free Krebs/HEPES Ringer solution (115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 25 mM HEPES [pH 7.4]) twice and pre-incubated at 37 °C for 30 min with the glucose-free Krebs/HEPES Ringer solution. Following this, the cells were incubated in Krebs/HEPES Ringer solution containing 1 mg/mL bovine serum albumin and 6.25, 12.5, 25, or 50 μg/mL of glucose in the presence or absence of CAMFs for 60 min. An aliquot of the supernatant was collected for ELISA. The amount of insulin released was measured with a Mouse Insulin ELISA kit (Mercodia, Uppsala, Sweden) according to the manufacturer protocol. Results (in pmol) are expressed as the means ± SE of at least two independent experiments.

2.2.6. Western blotting analysis

To determine protein expression, 1×10^6 cells/mL were seeded onto 25-cm^2 tissue culture flasks. Cells were treated with 6.25, 12.5, or 25 μg/mL of CAMFs for 24 h; whole cell extracts were prepared as described by Looi et al. (2011). Briefly, cells were collected and lysed in lysis buffer (20 mM Tris [pH 7.4], 250 mM NaCl, 0.1% Triton X-100, 2 mM EDTA, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 4 mM sodium orthovanadate, 1 mM DTT), and 60 μg of the protein was resolved on 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred to PVDF membranes (Millipore). The membranes were blocked with 5% non-fat dry milk in PBS-T (0.05% Tween 20) for 1 h at room temperature. Membranes were probed with primary goat anti-GLUT-1, GLUT-2, or GLUT-4 antibody followed by horseradish peroxidase (HRP)–conjugated secondary donkey anti-goat antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Protein-antibody complexes were detected with a ProteoQwest™ Colorimetric Western Blotting Kit with 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, St. Louis, MO, USA). To ensure equal loading, membranes were stripped and re-probed with mouse anti-β-actin antibody (Santa Cruz Biotechnology Inc.).

2.3. In vivo studies

2.3.1. Experimental animals

Sprague–Dawley rats (180–200 g) were obtained from the Animal Care Unit of the UM Medical Centre (Kuala Lumpur, Malaysia), and maintained under pathogen-free conditions in a temperature (23 ± 2 °C) and light-controlled (12-h light/dark cycle) room with 35–60% humidity in the animal housing unit. The animals were acclimatized for 10 days prior to the experiments and provided rodent chow and water *ad libitum*.

We performed the animal experiments in accordance with the animal experimentation guidelines issued by the UM Animal Care and Use Committee (Ethics Number: FAR/10/11/2008/AA[R]) and in accordance with the internationally accepted principles for laboratory animal use and care.

2.3.2. In vivo acute oral and intraperitoneal toxicity studies

CAMFs oral and intraperitoneal acute toxicity tests were determined according to the guidelines of the Organization for Economic Co-operation and Development (OECD). We used healthy adult Sprague Dawley rats of either sex. The rats were fasted overnight, divided into 12 groups ($n = 6$), and injected intraperitoneally (IP) or orally fed CAMFs at doses 10, 20, 50, 100 and 500 mg/kg body weight (bw), CAMFs was completely dissolved in distilled water and filtered before injecting i.p to the animals; the control groups were given distilled water alone. We observed the rats for 1 h continuously and then hourly for 4 h and finally after every 24 h up to 14 days for any physical signs of toxicity such as writhing, gasping, palpitation, and decreased respiratory rate or for any lethality.

2.3.3. Induction of DM1

We induced DM1 in overnight-fasted normal male rats through intraperitoneal administration of 65 mg/kg of freshly prepared STZ (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 M citrate buffer (pH 4.5) in a volume of 1 mL/kg bw. STZ-injected rats were given 20% glucose solution for 12 h to prevent initial drug-induced hypoglycemic mortality. Hyperglycemia was confirmed by elevation in blood glucose levels, determined at 96 h after the STZ administration. Rats with fasting blood glucose range of 22–26 mmol/L were considered DM1 and used for the study.

2.3.4. Induction of DM2

DM2 was induced (Masiello et al., 1998) with slight modifications, by using standardized dose of STZ. Intraperitoneal injection of freshly prepared STZ (55 mg/kg) in 0.1 M citrate buffer (pH 4.5) in a volume of 1 mL/kg was injected to overnight-fasted normal male rats, 15 min after i.p administration of nicotinamide (210 mg/kg). Hyperglycemia was confirmed by elevation in blood glucose levels, determined at 96 h after the STZ-nicotinamide administration. Rats with a fasting blood glucose range of

11–14 mmol/L were considered DM2 and subsequently used for the study.

2.3.5. Division of animals for type 1 and type 2 diabetic model study

The rats were divided into two segments: one each for the type 1 and type 2 diabetic model study. The type 1 segment was divided into the following groups, Group 1: normal control rats, Group 2: type 1 diabetic control rats, Group 3: type 1 diabetic rats treated with 6 U/kg of insulin (standard positive), Group 4: diabetic rats treated with 100 mg/kg of CAMFs, and Group 5: diabetic rats treated with 50 mg/kg of CAMFs. The type 2 segment was divided into the following groups, Group 1: normal control rats, Group 2: type 2 diabetic control rats, Group 3: type 2 diabetic rats treated with 50 mg/kg of glibenclamide (standard positive), Group 4: diabetic rats treated with 100 mg/kg of CAMFs, and Group 5: diabetic rats treated with 50 mg/kg of CAMFs. All groups were injected and fed once daily with the respective doses of CAMFs or drugs for 4 weeks (28 days). Administration was based on volume (2 mL/200 g bw). CAMFs was completely dissolved in distilled water and filtered before i.p. injection.

2.3.6. Experimental procedure

We collected blood samples from the tail by snipping; fasting blood glucose concentrations were determined weekly after the above treatments by a glucose oxidase-peroxidase enzymatic method using a standardized glucometer (Accu-Check Performa, Roche Diagnostic Germany). Changes in body weight, food intake, and water intake were recorded daily. After the 4-week treatment period, all groups from both segments were fasted overnight and sacrificed by cervical dislocation under mild anesthesia. Blood was collected upon decapitation into heparinized tubes and centrifuged at 2000 rpm for 10 min, the serum was collected and stored at $-80\,^{\circ}C$ until analysis.

2.3.7. Glucose challenge (glucose tolerance test)

We used the oral glucose tolerance test (OGTT) to evaluate the effectiveness of CAMFs that had demonstrated the highest glycemic control as studied in the type 1 and type 2 diabetic rat models described earlier. CAMFs (100 or 50 mg/kg) was administered to overnight-fasted rats in 2 mL/200 g bw doses. Fasting blood glucose concentrations were measured before the respective CAMFs administrations, following which oral glucose (3 g/kg) was administered and blood glucose levels were measured at 30, 60, 90, and 120 min, respectively.

2.3.8. Estimation of insulin and body weight

We determined serum insulin by using radioimmunoassay kit (Packard, USA) according to the manufacturer protocol. In addition, the whole body weight of type 1 and type 2 diabetic rats were examined at the end of study period in normal, treated, and untreated diabetic rats.

2.4. Statistical analysis

The results are expressed as mean ± standard deviation (SD). Significant differences between the means of the experimental groups were identified with analysis of variance (ANOVA), followed by the Tukey–Kramer multiple comparisons test (GraphPad version 5.0; GraphPad Software Inc., San Diego, CA, USA). Statistical significance was set at $P<0.001$, $P<0.01$, $P<0.05$. We performed log IC_{50} calculations using the built-in algorithms for dose–response curves with variable slope. We set a fixed maximum value of the dose–response curve to each sample's maximum obtained value.

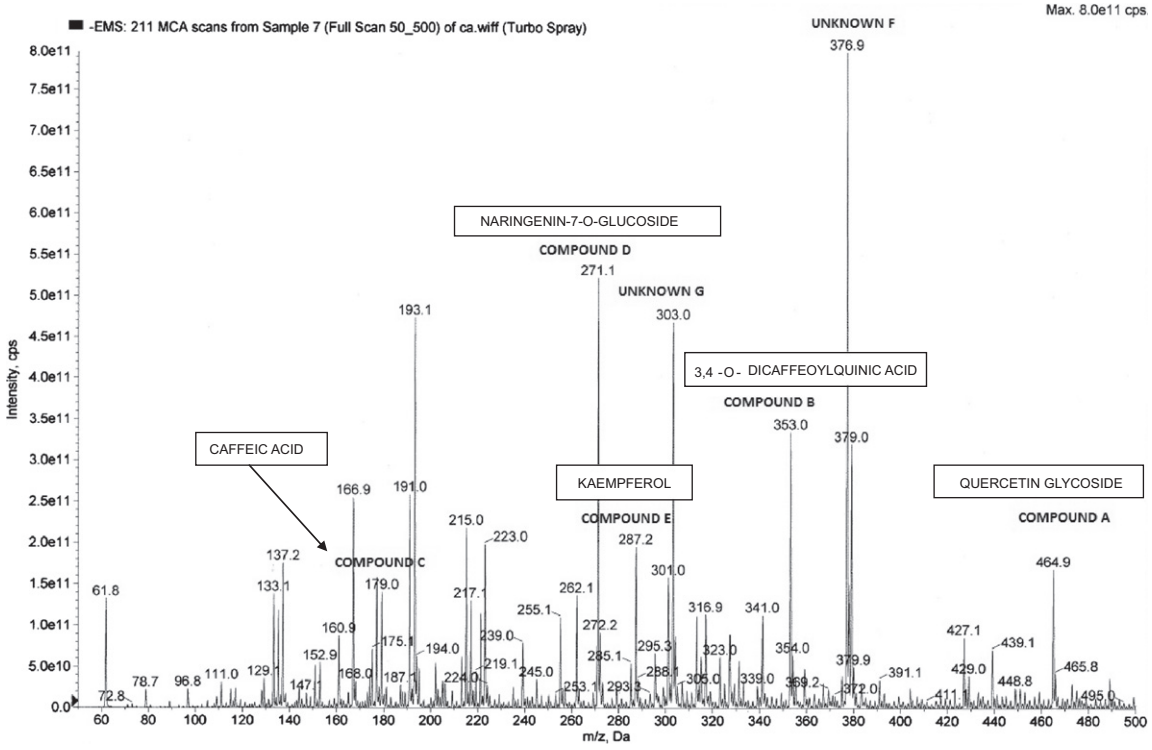

Fig. 1. The mass spectrometric main (parent) chromatogram of CAMFs indicates the presence of characterized compounds on the basis of their mass fragmentation pattern as (compound A) quercetin glycoside, (compound B) 3,4-*O*-dicaffeoylquinic acid, (compound C) caffeic acid, (compound D) naringenin-7-*O* glucoside, and (compound E) kaempferol with other unknown compounds.

3. Results

3.1. Fraction yield and LCMS-MS analysis

The final yield of the obtained CAMFs fractions, was 12.6% w/w. Phytochemical analysis using LCMS-MS demonstrated the presence of quercetin glycoside, 3,4-*O*-dicaffeoylquinic acid, caffeic acid, naringenin-7-*O* glucoside, and kaempferol, as the major compounds in CAMFs based on the main peaks selected, as well as other unknown compounds (Fig. 1). The fragmentation patterns of known compounds were in agreement with those documented in the literatures. The mass spectrometric characterization of Compound A indicates the presence of quercetin glycosides (Lin and Harnly, 2007). The fragmentation pattern of Compound B displays an ion peak at *m/z* 515, indicating the loss of the first caffeoyl, loss of the second caffeoyl at *m/z* 353, and loss of the third caffeoyl at *m/z* 191 to yield quinic acid, a loss of caffeic acid at *m/z* 179, and another caffeoyl at *m/z* 135. Thus, the compound was characterized as 3,4-*O*-dicaffeoylquinic acid (Lin and Harnly, 2008; Gouveia and Castilho, 2011). Compound C exhibits fragment ions at *m/z* 179 and *m/z* 135, the characteristic ions of caffeic acid (Luo et al., 2003; Lin and Harnly, 2008; Plazonic et al., 2011). Compound D was identified as naringenin-7-*O*-glucoside based on mass fragment ions at *m/z* 433 and at *m/z* 271 and 153 (Charrouf, et al., 2007). (E) Compound E was identified as kaempferol based on mass fragments at *m/z* 287 and *m/z* 151(Sun et al., 2007).

3.2. GC–MS analysis

The possible chemical composition in CAMFs analyzed by GC/MS is presented in Table 1. Major peaks were selected and identified on the basis of similarity index. The most abundant components determined are decanoic acid, dodecanamide, pentadecanoic acid, 14-methyl-methyl ester, tetradecanamide, hexadecanoic acid, ethyl ester, octadecanoic acid, and ethyl ester. Compounds with lower percentage (less than 80 similarity index, SI) were considered as minor constituents and these compounds were excluded from the list.

3.3. Effect of CAMFs on β-TC6 cell proliferation

To evaluate whether CAMFs is cytotoxic towards β-TC6 cells, we apply various concentrations of CAMFs and cell viability was analyzed using MTT assays 24 h after treatment. Fig. 2a illustrates the dose–response curves in the end-point cytotoxicity assay. CAMFs concentrations of up to100 μg/mL did not exert cytotoxic effects on β-TC6 cell viability. Next we monitored treated β-TC6 cells for 3 days using Xcelligence Real-time Cell Proliferation (RTCA) assays. RTCA data showed that β-TC6 cells treated with 50, 25, 12.5, 6.25 or 3.125 μg/mL of CAMFs proliferated in a similar manner compared to control cells throughout the 72-h treatment, as shown by an increase in the CI values, (Fig. 2b). However, high-concentration treatment (100 μg/mL) decreased the CI values after 72 h, suggesting that high concentration of CAMFs may inhibit β-TC6 cell proliferation after prolonged treatment. The IC_{50} values of CAMFs on β-TC6 cells at 24, 48, and 72 h were 518, 416, and 94 μg/mL, respectively.

3.4. Effect of CAMFs on 2-NBDG uptake

Fig. 3 illustrates the endocytosis of the fluorescent glucose analog 2-NBDG by mouse β-TC6 cells and increased intracellular 2-NBDG fluorescence is visible in distinct groups of cells. Fluorescence appeared to be restricted to the cytoplasm. As the duration defined for 2-NBDG uptake would have been sufficient for the stabilization of 2-NBDG uptake and metabolism,

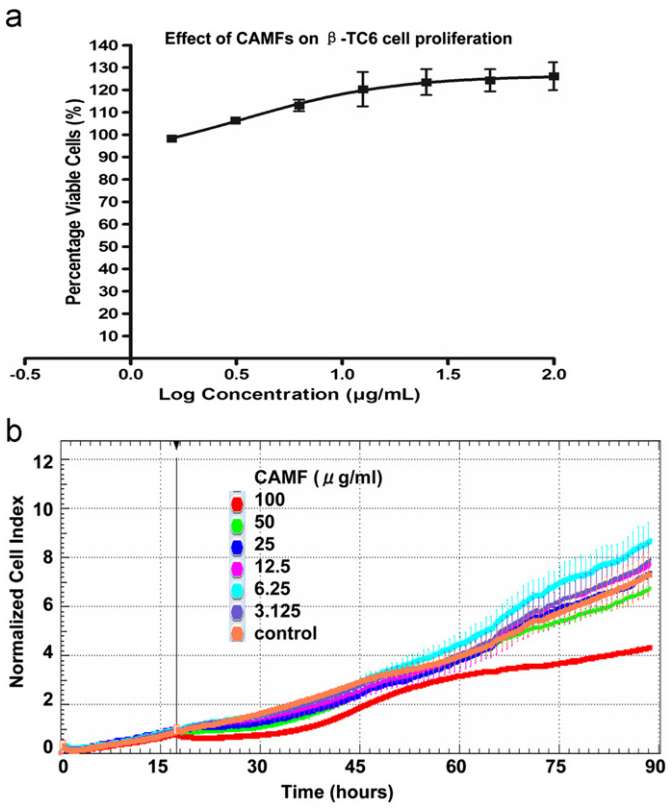

Fig. 2. (a) MTT assay growth dose–response curves of CAMFs tested on mouse β-TC6 cells. (b) Dynamic monitoring of cell proliferation using RTCA. β-TC6 cells were seeded in a 16X E-plate device and continuously monitored up to 90 h after treatment with various concentrations of CAMFs. CI values were normalized to the time point of CAMFs addition, indicated by the vertical black line.

Table 1
Compounds tentatively identified in CAMFs.

Peak No.	RT[a]	Compounds[b]	SI[c]	MW	Molecular Formula
1	9.40	Decanoic acid, ethyl ester	95	200	$C_{12}H_{24}O_2$
2	11.57	Tetradecanamide	86	227	$C_{14}H_{29}NO$
3	12.41	Octadecanamide	81	281	$C_{18}H_{35}NO$
4	13.10	Dodecanamide	93	287	$C_{16}H_{33}NO_3$
5	16.30	Pentadecanoic acid, 14-methyl-methyl ester	91	270	$C_{17}H_{34}O_2$
6	17.70	Hexadecanoic acid, ethyl ester	86	284	$C_{18}H_{36}O_2$
7	20.80	Octadecanoic acid, ethyl ester	81	312	$C_{20}H_{40}O_2$

[a] RT, Retention time (min).
[b] Compounds listed according to their relative area percentage (peak area relative to the total peak area percentage.
[c] SI, Similarity index.

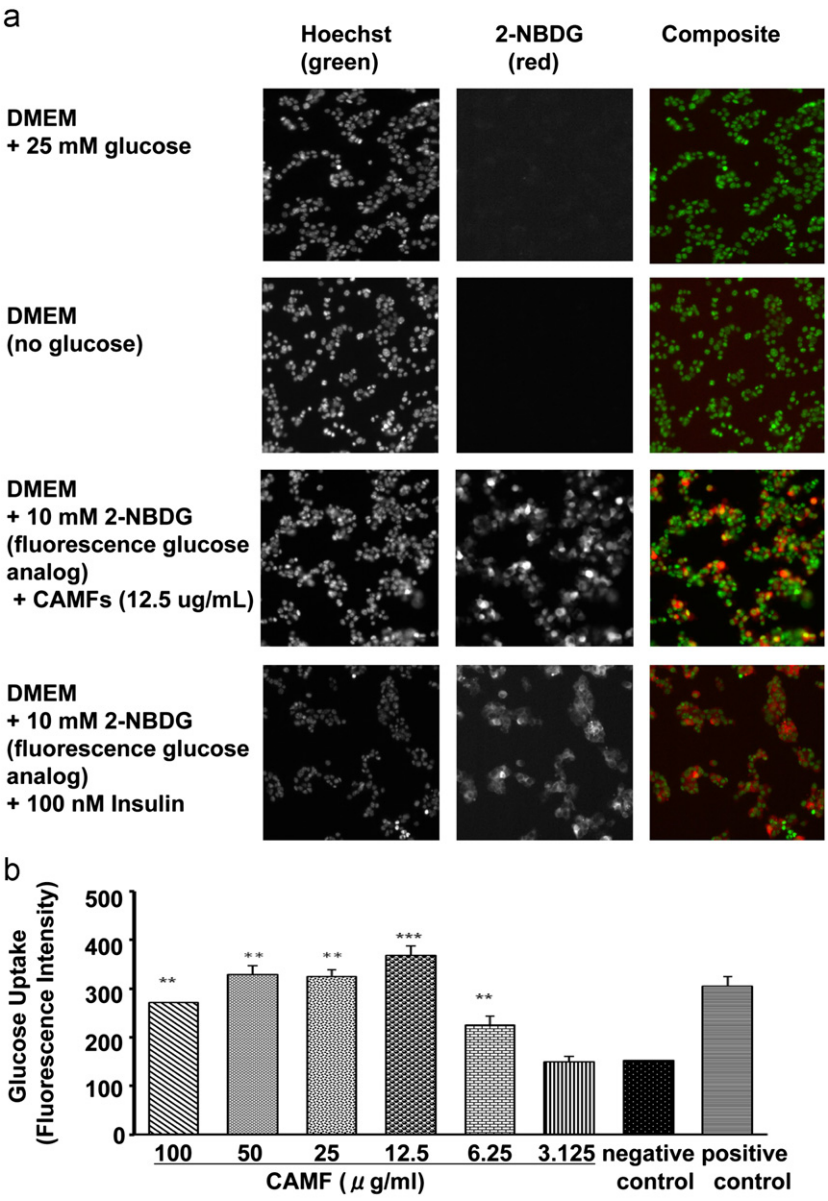

Fig. 3. (a) Fluorescence images of β-TC6 cells exposed to 10 mM 2-NBDG for 30 min revealing heterogeneous 2-NBDG uptake and metabolic activity. The cells were imaged after a 10-min washout of 2-NBDG. (b) Glucose uptake in β-TC6 cells after 60-min exposure to 10 mM 2-NBDG and CAMFs. Significant difference compared to negative control ($^{**}P < 0.01$ or $^{***}P < 0.001$).

this heterogeneous intracellular fluorescence suggests that β-TC6 cells retain the heterogeneous glucose uptake activity of native β-cells.

3.5. Effect of CAMFs on insulin secretion

Fig. 4 displayed the result of insulin secretion. In the absence of glucose, insulin secretion was low in the β-TC6 cells. However, CAMFs increased insulin secretion in a dose-dependent manner at glucose concentrations of 6.25, 12.5, 25, and 50 mM). In particular, at 12.5 μg/mL CAMFs elicited a marked increase in insulin secretion in the β-TC6 cells ($^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$).

3.6. Effect of CAMFs on glucose transporter proteins

At 24 h after CAMFs treatment, the protein level of GLUT-1, GLUT-2 and GLUT-4 in β-TC6 cells were examined by Western blotting analysis. As presented in Fig. 5a–c, CAMFs increased GLUT-2 and GLUT-4 protein levels in a dose-dependent manner compared to control ($^{*}P < 0.05$ or $^{**}P < 0.01$). In contrast, CAMFs did not alter GLUT-1 protein level (Fig. 5a).

3.7. Acute oral and intraperitoneal toxicity study

The acute oral and i.p toxicity studies revealed CAMFs non-toxic nature; no lethality or toxic reactions were observed at any of the doses tested. Based on these findings, 100 mg/kg and 50 mg/kg doses were chosen as the maximum doses for further experiments involving i.p and oral routes of administration, respectively.

3.8. Effect of CAMFs on blood glucose levels of type 1 diabetic rats

Blood glucose levels were evaluated weekly in normal, CAMFs-treated, and untreated diabetic rats, the outcomes of which are listed in Table 2. The standard drug insulin (6 U/kg) produced

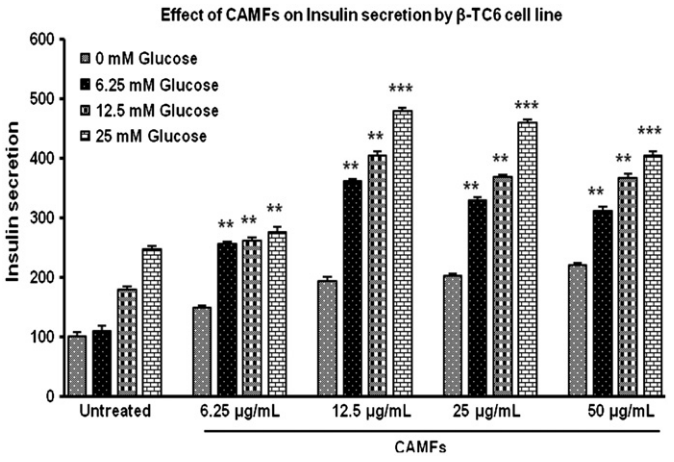

Fig. 4. Insulin release elicited by CAMFs in β-TC6 cells. The cells were incubated in Krebs/HEPES buffer (pH 7.4) containing no glucose or 6.25, 12.5, or 25 mM glucose in the absence or presence of CAMFs for 60 min at 37 °C.

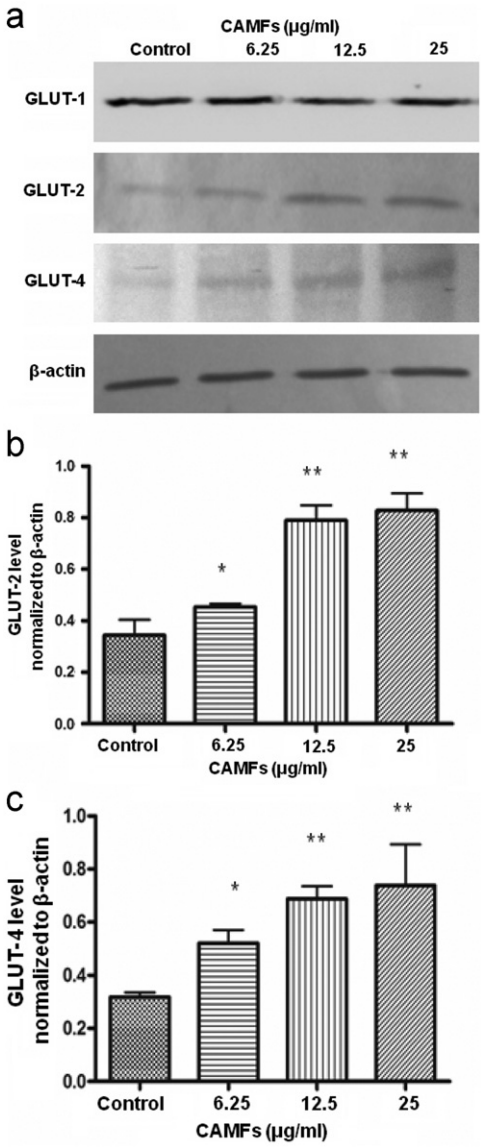

Fig. 5. (a) Western blot analysis of GLUT-1, GLUT-2 and GLUT-4 expression in control and CAMFs-treated samples. Membranes were stripped and reprobed with β-actin. Histogram (b and c) denotes band intensity ratio of GLUT-2 and GLUT-4 after normalization with β-actin in non-treated β-TC6 cells.

significant decrease in blood glucose levels, compared to untreated diabetic rats, starting from week 1 and produced maximal fall of 64.08% after end of the study period (4-week). While administration of 100 and 50 mg/kg of CAMFs demonstrated significant reduction in blood glucose levels at week-4 when compared with untreated diabetic rats, and exhibited decreases of 23.21% and 21.05% after 4-week treatment period. The blood glucose levels of normal rats were not changed, whereas in the untreated diabetic rat blood glucose levels were significantly and continuously elevated, until end of the study period.

3.9. Effect of CAMFs on blood glucose levels of type 2 diabetic rats

The results of the weekly blood glucose evaluations in normal, CAMFs-treated, and untreated diabetic rats are presented in Table 3. The blood glucose levels of normal rats were not altered until the end of the study period, while that in the untreated diabetic rats were significantly elevated. Oral administration of 100 and 50 mg/kg of CAMFs and 50 mg/kg of glibenclamide produced a significant reduction in blood glucose levels at weeks 1–4 when compared with untreated diabetic rats. In the final week of study period, 100 mg/kg of CAMFs produced the maximal decrease of blood glucose level (51.40%) as compared to untreated diabetic rats, and 50 mg/kg of CAMFs caused 46.47% decline, whereas glibenclamide group achieved 50% drop in blood glucose levels.

3.10. Effect of CAMFs on OGTT of type 2 diabetic rats

Result of OGTT is showed in Table 4. In diabetic rats, CAMFs at 100 and 50 mg/kg demonstrated a significant reduction in blood glucose levels after 60-min of glucose load, compared to untreated diabetic rats. Similarly, after 90 min the blood glucose levels were continuously reduced to 66.31% and 52.94% after CAMFs treatment, whereas the glibenclamide-treated group caused (60.96%) reduction, respectively. This was followed by a reduction as great as 67.59% and 54.74% following the respective CAMFs doses at 120 min, whereas glibenclamide showed 62.01% markdown in the blood glucose levels.

3.11. Effect of CAMFs on insulin level and body weight of type 1 and type 2 diabetic rats

Fig. 6a illustrates the insulin levels in the experimental animals. In type 1 and type 2 groups, untreated diabetic rats exhibited significant reduction of insulin in the serum, compared to the normal control rats. Upon daily treatment of standard positive and 100 or 50 mg/kg bw of CAMFs to diabetic rats for 4 weeks, demonstrated significant improvement in insulin levels of type 2 diabetic rats as compared to untreated diabetic rats, whereas type 1 diabetic rats did not produce any significant sign of improvement.

Moreover, we observed a significant loss in the body weight of untreated type 1 and type 2 diabetic rats, as compared to normal control rats after 4 weeks of study period (Fig. 6b). While daily intake of food and water was significantly increased only in type 2 diabetic rats (Fig. S1a and S1b). Standard positive and CAMFs treatment significantly increased the body weight of type 2 diabetic rats compared to untreated diabetic rats. In contrast, only 100 mg/kg of CAMFs resulted in elevation of body weight in type 1 diabetic rats.

Table 2
Effects of CAMFs on fasting blood glucose level of type 1 diabetic rats.

Group	Fasting blood glucose level (mmol/L)				
	Pretreatment week	Treatment weeks			
	Week 0	Week 1	Week 2	Week 3	Week 4
Normal control	4.2 ± 0.13	4.4 ± 0.26	3.9 ± 0.18	3.6 ± 0.22	4.1 ± 0.32
Diabetic control	23.8 ± 1.19	25.6 ± 1.41	27.5 ± 1.24	28.7 ± 1.77	32.3 ± 2.69
Insulin (6 U/kg)	24.6 ± 1.21	9.9 ± 0.67[a (61.32)]	9.1 ± 0.97[a (66.90)]	10.4 ± 1.16[a(63.76)]	11.6 ± 1.36[a(64.08)]
CAMFs (100 mg/kg)	24.9 ± 0.94	23.8 ± 1.08[(7.03)]	22.2 ± 1.37[(19.27)]	23.1 ± 1.08[(19.51)]	24.8 ± 1.27[a(23.21)]
CAMFs (50 mg/kg)	25.2 ± 0.67	23.3 ± 0.88[(8.98)]	23.7 ± 0.97[(13.81)]	24.6 ± 1.21[(14.28)]	25.5 ± 1.03[a(21.05)]

Values denote mean ± SD, $n=6$.

[a] Represents statistical significance compared to diabetic control ($P<0.05$).
Values given in parenthesis represent percent decline in the blood glucose level in comparison with diabetic control group within the same week.

Table 3
Effects of CAMFs on fasting blood glucose level of type 2 diabetic rats.

Group	Fasting blood glucose level (mmol/L)				
	Pretreatment week	Treatment weeks			
	Week 0	Week 1	Week 2	Week 3	Week 4
Normal control	4.0 ± 0.21	4.2 ± 0.19	4.4 ± 0.25	4.3 ± 0.34	4.1 ± 0.29
Diabetic control	11.8 ± 0.57	12.1 ± 0.91	12.9 ± 1.04	13.7 ± 1.17	14.2 ± 1.37
Glibenclamide (50 mg/kg)	11.9 ± 0.65	7.6 ± 0.32[a(37.19)]	7.4 ± 0.43[a(42.63)]	7.5 ± 0.66[a(45.25)]	7.1 ± 0.36[a(50.00)]
CAMFs (100 mg/kg)	12.2 ± 0.56	7.8 ± 0.44[a(35.53)]	7.7 ± 0.51[a(40.31)]	7.3 ± 0.33[a(46.71)]	6.9 ± 0.49[a(51.40)]
CAMFs (50 mg/kg)	12.7 ± 0.71	8.4 ± 0.39[a(30.57)]	8.9 ± 0.39[a(31.00)]	8.1 ± 0.46[a(40.87)]	7.6 ± 0.53[a(46.47)]

Values denote mean ± SD, $n=6$.

[a] Represents statistical significance compared to diabetic control ($P<0.05$).
Values given in parenthesis represent percent decline in the blood glucose level in comparison with diabetic control group within the same week.

Table 4
Effects of CAMFs on fasting blood glucose level of type 2 diabetic rats after glucose load.

Group	0 min	30 min	60 min	90 min	120 min
Normal control	3.9 ± 0.17	7.9 ± 0.25	7.1 ± 0.29	6.3 ± 0.32	5.1 ± 0.41
Diabetic control	15.3 ± 0.52	22.2 ± 0.64	19.2 ± 0.4	18.7 ± 0.53	17.9 ± 0.35
Glibenclamide (50 mg/kg)	7.3 ± 0.39	9.8 ± 0.23[a(55.85)]	8.1 ± 0.42[a(57.81)]	7.3 ± 0.59[a(60.96)]	6.8 ± 0.44[a(62.01)]
CAMFs (100 mg/kg)	6.8 ± 0.26	9.5 ± 0.37[a(57.20)]	7.3 ± 0.31[a(61.97)]	6.3 ± 0.28[a(66.31)]	5.8 ± 0.48[a(67.59)]
CAMFs (50 mg/kg)	8.2 ± 0.71	10.4 ± 0.43[a(53.15)]	8.8 ± 0.47[a(54.16)]	8.8 ± 0.35[a(52.94)]	8.1 ± 0.29[a(54.74)]

Values denote mean ± SD, $n=6$.

[a] Represents statistical significance compared to diabetic control ($P<0.05$).
Values given in parenthesis represent percent decline in the blood glucose level in comparison with diabetic control group in that time period.

4. Discussion

Centratherum anthelminticum seeds are commonly used in India to treat diabetes. The seed extracts are available in Ayurvedic formulation for treating diabetes and various disorders. However, the anti-diabetic potential and the mechanism of CAMFs have not been thoroughly investigated. In the present study, we demonstrated that CAMFs exhibited potential anti-diabetic effects on pancreatic β-TC6 cells. Moreover, our *in vivo* results indicate that CAMFs possess glibenclamide-like activities in type 2 diabetic rats with lesser effects seen in type 1 diabetic rats.

Both MTT assays and RTCA results showed that CAMFs is not toxic to β-TC6 cells at 50 μg/ml, which is in agreement with the *in vivo* acute toxicity studies. We further investigated the effect of CAMFs on glucose uptake and insulin secretion by β-TC6 cells. Our results revealed that CAMFs dose-dependently stimulates glucose uptake and enhances insulin secretion in β-TC6 cells. The fluorescent glucose analog 2-NBDG has been used to determine cell viability as well as to estimate glucose uptake rates in a variety of cell types (Poitout et al., 1995; Yoshioka et al., 1996; Yamada et al., 2000; Leira et al., 2002; Zou et al., 2005; Yamada et al., 2007). β-TC6 cell is a β cell derivative, which can endocytose the glucose analog 2-NBDG as shown by increasing intracellular fluorescence in the cytoplasmic regions after CAMFs treatment. In addition, we detected higher insulin secretion in these cells, corresponding with enhanced 2-NBDG glucose uptake. These observations on β-TC6 cell lines clearly establish the potent ability of CAMFs in increasing insulin release and glucose uptake.

Following characterization of CAMFs effects in promoting glucose uptake and insulin secretion, we studied the most crucial factor in the insulin-signaling cascade, glucose transporter proteins, which are essential transporters responsible for the translocation of insulin-regulated glucose into the cells. Our results showed that CAMFs treatment increased GLUT-2 and GLUT-4 protein expression without any significant changes in GLUT-1 protein level compared with the control group. GLUT-4 is classically referred as "insulin-responsive transporter" and increased GLUT-4 expression may result in higher sensitivity to insulin which subsequently potentiates the influx of glucose into the cell (Suzuki and Kono, 1980; Slot et al., 1991; Kraegen et al., 1993). Whereas GLUT-2 is a transmembrane carrier protein mainly found in pancreatic β-cells. Increased GLUT-2

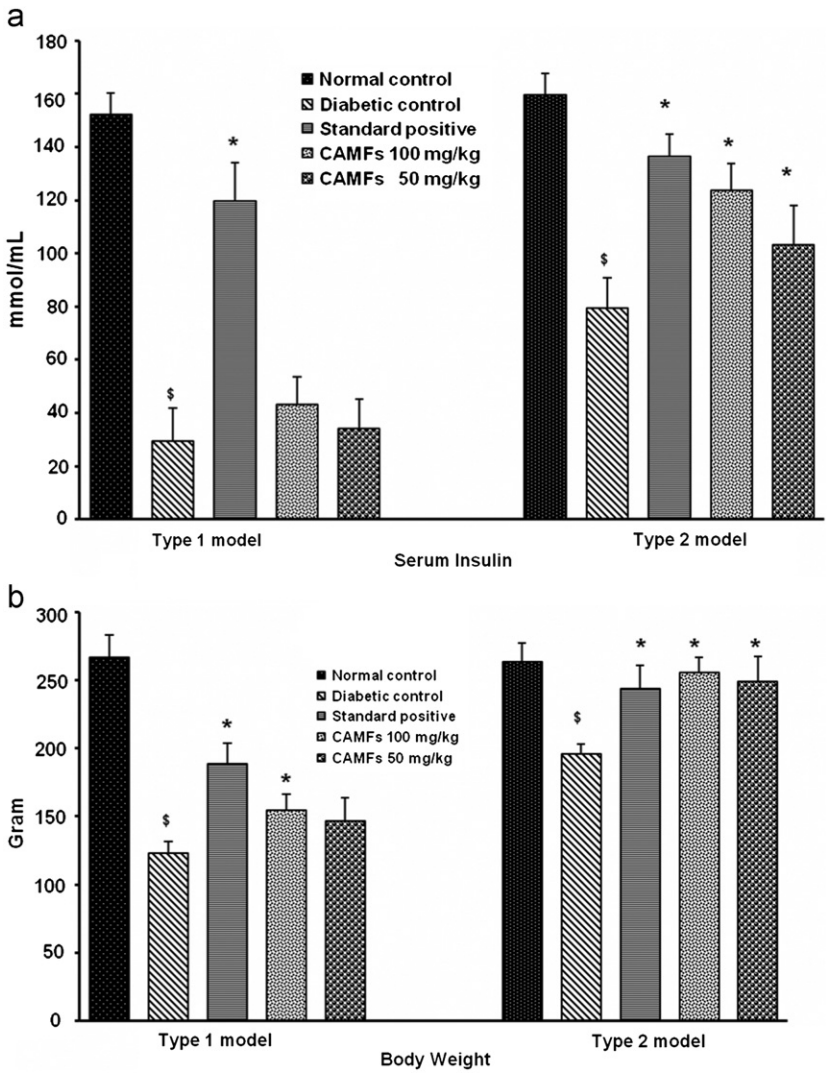

Fig. 6. Effects of CAMFs on insulin level and body weight of type 1 and type 2 diabetic rat models after 28 days (4 weeks) in comparison with diabetic and normal control rats. At the end of the treatment, rats were fasted for 12 h and blood was drawn to collect the serum for insulin measurement. Panel denotes (a) serum insulin and (b) whole body weight. The data are presented as means ± SD ($n=6$). $^{\$}$Significant difference compared to the normal control group ($P<0.05$). *Significant difference compared to the diabetic control ($P<0.05$).

expression is thought to play a constitutive role and responsible for higher glucose uptake in β-cells (Hyo-Sup et al., 2008). In contrast, loss of GLUT-2 may accompany the onset and contribute to the pathogenesis of insulin-dependent (DM1) and non-insulin-dependent diabetes mellitus (DM2) (Bjornholm and Zierath, 2005). Thus, phytochemicals in CAMFs could stimulate insulin-signaling cascade by upregulating GLUT-2 and GLUT-4 expression, leading to increased glucose uptake in the pancreatic β-TC6 cells.

Based on these positive findings, we studied the anti-diabetic potential of CAMFs in the management of type 1 or type 2 diabetic rats. Daily administration of varying concentrations of CAMFs to both groups of animals for 4 weeks produced differential reduction in the blood glucose levels. We found that only type 2 diabetic rats exhibited marked reduction of blood glucose, with less effect observed in the type 1 diabetic rats. Although type 1 diabetic rats displayed significant reduction of blood glucose levels after second week of treatment period, the overall percentage declined were less significant compared to type 2 diabetic rats. This was probably caused by the severe destruction of pancreatic β-cells by STZ in type 1 diabetic rats as these rats had very low to non-detectable serum insulin levels that were not significantly improved upon treatment with CAMFs. On the other hand, in the type 2 diabetic rats, nicotinamide administration prior to STZ injection may have partially protected β-cells by scavenging nitric oxide (NO) and rescued the β-cells from STZ-induced apoptosis (Masiello et al., 1998). Thus, the beneficial effects of CAMFs treatment in type 2 diabetic rats was likely due to improved insulin release and glucose uptake in remnant β-cells. Moreover, elevated insulin secretion by CAMFs can promote conversion of inactive glycogen synthetase to the active form, which increases the conversion of blood glucose into glycogen by enhancing the glycolytic and glycogenic processes with concomitant decrease in glycogenolysis and gluconeogenesis (Mahomed and Ojewole, 2003; Andrade-Cetto and Wiedenfeld, 2004).

In the present study, we observed significant loss in the body weight of type 1 and type 2 diabetic rats, a symptom synonymous with diabetes mellitus (Pupim et al., 2005). The characteristic loss of body weight associated with STZ-induced diabetes could be due to dehydration and catabolism of fats or breakdown of tissue proteins, which leads to wasting of muscle. CAMFs treated type 2 diabetic rats exhibited comparable increases in body weight. This could be the result of increased glucose uptake, insulin secretion and decreased fasting blood glucose levels, indicating improved glycemic control in the rats. Such findings of improvement in body weight amongst diabetic animals are consistent with treatment of other medicinal

plants reported to have potential anti-diabetic effects (Pari and Saravanan, 2004; Nagarajan et al., 2005).

In this study, we have scientifically verified the traditional basis for the use of *C. anthelminticum* seeds in diabetes mellitus. The anti-diabetic potential of CAMFs may be attributed to the polyphenolic constituents as identified by LCMS-MS including quercetin glycoside, 3,4-*O*-dicaffeoylquinic acid, caffeic acid, naringenin-7-*O*-glucoside and kaempferol. These dietary polyphenols possess wide therapeutic benefits and many researchers have demonstrated the anti-diabetic, anti-oxidant, and anti-inflammatory activities with these compounds (Jung et al., 2006; Ortiz-Andrade et al., 2007; Shih et al., 2012). In addition, decanoic and pentadecanoic acid which are characterized by GC–MS with high similarity index in CAMFs have previously been shown as potential modulating ligands for peroxisome proliferator-activated receptors (PPARs) (Malapaka et al., 2012). Thus, combination of these compounds in CAMFs may exert synergistic anti-diabetic effects in the type 2 diabetic rats.

5. Conclusions

In summary, the present findings suggest that the anti-diabetic effects of CAMFs may be due to the enhancement of 2-NBDG glucose uptake, stimulation of insulin secretion and inducing higher GLUT-2 and GLUT-4 transporter protein expression in β-TC6 cells. These observations were corroborated by the beneficial effects of CAMFs in attenuating hyperglycemia and augmenting insulin secretion in type 2 diabetic rat model. These findings supports the use of CAMFs as a potential adjunct dietary treatment of insulin-resistant type 2 diabetes and a potential source for the discovery of new orally active agent(s) for future therapy of diabetes.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2012.08.014.

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The methanolic fraction of *Centratherum anthelminticum* seed downregulates pro-inflammatory cytokines, oxidative stress, and hyperglycemia in STZ-nicotinamide-induced type 2 diabetic rats

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ABSTRACT

This study aimed to ascertain the potential of *Centratherum anthelminticum* seeds methanolic fraction (CAMFs) for the management of type 2 diabetes and its associated complications. CAMFs was initially tested on β-TC6 cells for H_2O_2-induced nuclear factor-κB (NF-κB) translocation effects. The result displayed that CAMFs significantly inhibited NF-κB translocation from cytoplasm into the nucleus, dose-dependently. Furthermore, a 12-week sub-chronic CAMFs study was carried out on streptozotocin (STZ)-nicotinamide–induced type 2 diabetic rat model to evaluate glycemia, essential biochemical parameters, lipid levels, oxidative stress markers, and pro-inflammatory cytokines level. Our study result showed that CAMFs reduced hyperglycemia by increasing serum insulin, C-peptide, total protein, and albumin levels, significantly. Whereas, elevated blood glucose, glycated hemoglobin, lipids and enzyme activities were restored to near normal. CAMFs confirmed antioxidant potential by elevating glutathione (GSH) and reducing malondialdehyde (MDA) levels in diabetic rats. Interestingly, CAMFs down-regulated elevated tumor necrosis factor α (TNF-α), interleukin (IL)-1β and IL-6 in the tissues and serum of the diabetic rats. We conclude that CAMFs exerted apparent antidiabetic effects and demonstrated as a valuable candidate nutraceutical for insulin-resistant type 2 diabetes and its associated complications such as dyslipidemia, oxidative stress, and inflammation.

1. Introduction

Despite the great strides that have been made in the understanding and management of diabetes, the incidence of the disease and its complications are increasing unabated. A combination of insulin resistance and an inadequate compensatory insulin secretory response accounts for non–insulin-dependent type 2 diabetes mellitus (DM2). It is the most prevalent disease in the world, affecting 7% of the population, or 285 million people worldwide. If untreated, DM2 may lead to insulin-dependent type 1 diabetes.

Clinical, preclinical, and epidemiological studies indicate an association between oxidative stress and inflammation in the development of DM2 and its complications (Zozulinska and Wierusz-Wysocka, 2006). In DM2, production of reactive oxygen species (ROS) is increased due to insulin resistance and hyperglycemia (Brownlee, 2001). Compared to healthy subjects, DM2 patients have a lower ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG), a major endogenous antioxidant. In contrast, malondialdehyde (MDA), a highly toxic by-product generated partially by lipid oxidation and ROS, is increased in patients with diabetes (Evans, 2007). The generated ROS create oxidative stress and exert major effects on signaling pathways, which further affect cellular metabolism and trigger a low-grade inflammatory reaction (Dominiczak, 2003). Lipid accumulation in adipose tissue and expansion of the fat mass in the liver initiate steatosis that

Abbreviations: γ-GT, γ-glutamyl transpeptidase; ALP, alkaline phosphatase; ALT, alanine transaminase; ANOVA, analysis of variance; AST, aspartate transaminase; ATCC, American Type Culture Collection; bw, body weight; CA, *Centratherum anthelminticum*; CAMFs, crude methanolic fraction of C. anthelminticum seeds; CRP, C-reactive protein; DM2, type 2 diabetes; DMEM, Dulbecco's modified Eagle medium; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; FBS, fetal bovine serum; FFA, free fatty acid; GSSG, oxidized glutathione; HbAlc, glycated hemoglobin; HCS, high content screening; HDL-C, high-density lipoprotein cholesterol; IKK-β, NF-κB regulatory protein kinase; IL, interleukin; IRS, insulin receptor substrate; LCMS–MS, liquid chromatography–tandem mass spectrometry; LDL-C, low-density lipoprotein cholesterol; NMR, nuclear magnetic resonance; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PKC, protein kinase C; ROS, reactive oxygen species; SD, standard deviation; SH, sulfhydryl; SMc, smooth muscle cell; STZ, streptozotocin; TC, total cholesterol; TG, triglycerides; TNB, 5-thio-2-nitrobenzoic acid.

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promotes low-grade inflammation via activation of nuclear factor-κB (NF-κB) (Arkan et al., 2005) and provokes an inflammatory process accompanied by local production and secretion of pro-inflammatory cytokines and chemokines (Hotamisligil et al., 1995; Jager et al., 2007). It has been hypothesized that DM2 is a manifestation of an ongoing acute-phase response that is primarily characterized by alterations of the so-called acute-phase proteins, such as C-reactive protein (CRP) (Pickup and Crook, 1998; Pickup et al., 1997), with other cytokines that are central mediators of inflammatory reactions, such as interleukin (IL) 6, IL-1β, or tumor necrosis factor α (TNF-α). It is well established that cytokines operate as a network in stimulating the production of acute-phase proteins. For example, the effects of IL-6 on CRP synthesis largely depend on its interaction with IL-1β (Joachim, 2003). The pro-inflammatory cytokine TNF-α reduces insulin sensitivity in muscle tissue and stimulates hepatic lipogenesis and hyperlipidemia (Franckhauser et al., 2008). However, it appears that treatments aimed at reducing the degree of oxidative stress and the production of pro-inflammatory cytokines in DM2 is warranted.

Centratherum anthelminticum (L.) Kuntze (bitter cumin) is a member of the Asteraceae family, an important plant of great significance and usage in Ayurvedic medicine. The records from traditional healers and ethno-botanists state that it is useful in alleviating diabetes. Experimental studies have proven the pharmacological potential of this plant in diverse biological activities, some of which are anti-diabetic, anti-cancer with anti-oxidant and anti-inflammatory activity (Ani and Naidu, 2008; Fatima et al., 2010; Arya et al., 2012a,b,c). Nevertheless, researchers have yet to investigate the hypoglycemic action of sub-chronic administration of *C. anthelminticum* seeds defatted crude methanolic fraction (CAMFs) or the plant's other healing properties, some of which might act against other inflammatory processes and oxidative stress associated with DM2.

Therefore, we attempted to gain a better understanding of the effect of CAMFs on ROS-induced oxidative stress associated with insulin resistance signaling pathway in H_2O_2-induced NF-κB activation on mouse pancreatic β-TC6 cells. Subsequently, we carried out *in vivo* studies to determine whether long-term administration of CAMFs for 12-weeks exerts anti-hyperglycemic, anti-hyperlipidemic, anti-oxidant, and inflammatory cytokines inhibitory effects in STZ-nicotinamide–induced type 2 diabetic rats.

2. Materials and methods

2.1. Preparation of CAMFs

2.1.1. Collection of plant material

Dried *C. anthelminticum* seeds were procured from the medicinal plant cultivation zone of Amritum Bio-Botanica Herbs Research Laboratory Pvt. Ltd., (Madhya Pradesh, India) in April 2008. The seeds were botanically classified and authenticated by the company's quality control department. Voucher specimens (CA-9) were deposited with the company and with the Department of Pharmacology in the Faculty of Medicine at the University of Malaya.

2.1.2. Extraction and fractionation

Two kilogram of seeds were coarsely powdered and first extracted with 100% n-hexane using hot extraction with a Soxhlet extractor for 24 h. Further fractionation of the obtained defatted residue was carried out using 100% chloroform, and lastly with 100% (absolute) methanol. The solvents from each crude fractions were dried by rotary evaporation under reduced pressure at a maximal temperature of 40 °C. The final fraction was then freeze-dried to yield a crude methanolic fraction (CAMFs), that was stored at −20 °C until further use. Thereafter, CAMFs was subjected to mass spectometry analysis by using LCMS–MS, for the qualitative analysis of major compounds, as well as evaluated for the total phenolic and flavonoid contents.

2.1.3. Phytochemical analysis of CAMFs by LCMS–MS

Phytochemical analysis of the major compounds in CAMFs was carried out with liquid chromatography–tandem mass spectrometry (LCMS–MS). A triple quadrupole mass spectrometer equipped with a turbo ion spray source (AB Sciex QTrap 5500, Ontario, Canada) was used to obtain the MS/MS data in negative ion mode. The mobile phase consists of 0.1% formic acid in water and 0.1% formic acid in acetonitrile (ACN), were eluted by gradient elution at a flow rate of 0.4 mL/min with an injection volume of 20 μL. Separation of the compounds was performed using a Luna 3-μm RP C18 column (100 × 2.00 mm; Phenomenex). The turbo ion source settings were as follows: capillary voltage, −4000 V; dry gas flow (N_2), 9 L/min; nebulizer pressure, 35 psi; and capillary temperature, 365 °C. A full scan of the mass spectra was recorded from *m/z* 50 to *m/z* 1000. The acquisition data was processed with Analyst Software version 1.5.1. Compounds were characterized based on their UV spectra and MS^2 and MS^3 fragmentations spectra data by correlation with previous reports (Table 1). Whereas, compounds F, G, H, I and J in the table are unknown compounds.

2.1.4. Determination of total phenolic content

The total phenolic content in CAMFs was determined by adapting the method as published in our previous article (Arya et al., 2012a,b). In brief, CAMFs was initially prepared in methanol with concentration of 10 mg/mL. From this solution 5 μL was transferred to 96-well mircoplate (TPP, USA). To this, 80 μL of Folin–Ciocalteu reagent (1:10) were added and mixed thoroughly. After 5 min, 160 μL of sodium bicarbonate solution ($NaHCO_3$ 7.5%) were added and the mixture was allowed to stand for 30 min with intermittent shaking. Absorbance was measured at 765 nm using microplate reader (Molecular Devices, Sunnyvale, USA). The TPC was expressed as gallic acid equivalent (GAE) in mg/g fraction, obtained from the standard curve of gallic acid.

The gallic acid standard curve was established by plotting concentration (mg/mL) versus absorbance (nm) ($y = 0.001x + 0.045$; $R^2 = 0.9975$), where y is absorbance and x is concentration in GAE ($n = 3$).

2.1.5. Determination of total flavonoid content

The total flavonoid content in CAMFs was determined by following the method published in our previous article (Arya et al., 2012a,b). In brief, 5 mL of 2% aluminum trichloride was mixed with the same volume of CAMFs. Absorbance readings at 415 nm were taken after 10 min against a blank sample consisting of 5 mL of sample solution and 5 mL of methanol without aluminum trichloride. The total flavonoid content was determined using a standard curve of mg Quercetin (Q) equivalents. The average of three readings was used and then expressed as quercetin equivalents (QE) on a dry weight (DW) basis.

2.2. In vitro assay

2.2.1. Cell culture

Mouse pancreas β-TC6 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in 15% fetal bovine serum (FBS) in Dulbecco's Modified Eagle Medium (DMEM). Cultures were maintained at 37 °C in 5% CO_2 in a humidified incubator. The growth medium was changed every 3 days.

2.2.2. NF-κB translocation assay

We seeded 1.5×10^4 cells/mL onto a 96-well plate. The cells were pre-treated for 1 h with 6.25, 12.5, or 25 μg/mL CAMFs, or were left untreated. The cells were then stimulated for NF-κB translocation with 50 μM of H_2O_2 for 30 min. NF-κB staining was performed according to the manufacturer's instructions with an NF-κB activation kit (Cellomics Inc., Pittsburgh, PA, USA). We used the ArrayScan high content screening (HCS) system (Cellomics Inc., Pittsburgh, PA, USA) to quantify the difference between the intensity of nuclear and cytoplasmic NF-κB–associated fluorescence.

2.3. Preclinical studies

2.3.1. Experimental animals

We obtained Sprague–Dawley rats weighing 180–200 g from the Animal Care Unit of the University Malaya Medical Centre (Kuala Lumpur, Malaysia) and maintained them under pathogen-free conditions in the animal housing unit in a temperature (23 ± 2 °C) and light-controlled (12-h light/dark cycle) room with 35–60% humidity. The animals were acclimatized for 10 days prior to the experiments and were provided rodent chow and water *ad libitum*.

The animal experiments were performed in accordance with the guidelines for animal experimentation issued by the Animal Care and Use Committee at the University of Malaya (Ethics Number: FAR/10/11/2008/AA[R]) and was conducted in accordance with internationally accepted principles for laboratory animal use and care.

2.3.2. Oral acute toxicity studies

CAMFs oral acute toxicity tests were carried out according to the guidelines of the Organization for Economic Co-operation and Development (OECD). For these test, we used healthy adult Sprague Dawley rats of either sex (180–200 g). These rats were fasted overnight, divided into 6 groups ($n = 6$), and orally fed with CAMFs in doses of 10, 20, 50, 100 and 500 mg/kg; the control group was given distilled water. We observed the rats for 1 h continuously and then hourly for 4 h for any

Table 1
Characterization of phenolic compounds in CAMFs by LC–MS/MS.

	Tentative compounds	$[M-H]^-$ (*m/z*)	Major fragment ions (*m/z*)	UV λ_{max} (nm)	References
A	Quercetin glycoside $C_{21}H_{20}O_{12}$	463	301, 287, 271	220, 300	Lin and Harnly (2007)
B	3,4–0-Dicaffeoylquinic acid $C_{25}H_{24}O_{12}$	515	353, 191, 179, 135	240, 300	Gouveia and Castilho (2011) and Lin and Harnly (2008)
C	Caffeic acid $C_9H_8O_4$	179	135	220, 325	Luo et al. (2003), Lin and Harnly (2008) and Plazonic et al., 2011
D	Naringenin-7–0-glucoside $C_{21}H_{22}O_{10}$	433	271, 153	200, 300	Charrouf et al., (2007)
E	Kaempferol $C_{15}H_{10}O_6$	287	287, 151	220, 340	Sun et al. (2007)
F	Unknown	377	253, 215, 135	200,300	–
G	Unknown	303	181, 167, 135	220,300	–
H	Unknown	341	303, 167, 135	200,300	–
I	Unknown	539	503, 377, 221	200,300	–

changes in the blood glucose levels and finally after every 24 h up to 14 days for any physical signs of toxicity, such as writhing, gasping, palpitation and decreased respiratory rate or for any lethality.

2.3.3. Induction of DM2

DM2 was induced by following the methods of Masiello et al. (1998) with slight modifications. In brief, after standardization of STZ (Sigma-Aldrich, St. Louis, MO, USA) doses, single intraperitoneal injection of freshly prepared STZ (55 mg/kg b.wt) in 0.1 M citrate buffer (pH 4.5) in a volume of 1 ml/kg b.wt was injected to overnight fasted normal male rats, 15 min after i.p administration of nicotinamide (210 mg/kg). Hyperglycemia was confirmed by elevated blood glucose levels, determined at 96 h after the STZ-nicotinamide administration. Rats with fasting blood glucose range of 11–14 mmol/L were considered as type 2 diabetic and further used for the study.

2.3.4. Experimental procedure

The rats were divided into the following 6 groups (9 or 10 rats per group): normal control rats, diabetic control rats, diabetic rats treated with 50 mg/kg bw of glibenclamide (a standard drug), and diabetic rats treated with 50, 25, and 10 mg/kg bw of CAMFs, respectively. All groups were fed their respective doses of CAMFs or glibenclamide once daily for 12 weeks. After the 12-week treatment period, all 6 groups were fasted for 12 h and then anesthetized using pentobarbital; the blood was collected into heparinized tubes. Any residual blood was removed by perfusion using phosphate-buffered saline (PBS; pH 7.4) through the abdominal aorta. The blood was centrifuged at 2000 rpm for 10 min and the serum was collected and stored at −80 °C until analysis. The liver, kidney, and pancreas were removed, washed in ice-cold isotonic saline, and blotted individually on ash-free filter paper; the organs were weighed and tissues were collected and fixed in 10% formalin for histology and markers estimation. Tissues were then homogenized in ice-cold 5% metaphosphoric acid or PBS (pH 7.4). The homogenates collected were used for the enzyme estimations before being centrifuged at 4500 rpm for 30 min at 4 °C, and the supernatant was collected for the analysis of oxidative stress markers and pro-inflammatory cytokines.

2.3.5. Assessment of biochemical parameters

Glycemia in fasted animals with free access to water was quantified weekly from tail vein blood using a standardized glucometer (Accu-Chek; Roche, Mannheim, Germany) until the end of the treatment period, as well as intake of food was also monitored on daily basis. Serum insulin and C-peptide levels were measured using a radioimmunoassay kit (Packard, USA) according to the manufacturer protocol. Glycated hemoglobin (HbA1c) was estimated by a DCA 2000 device (Bayer, Sunnyvale, CA, USA). Serum total protein, albumin and lipids, i.e., triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and free fatty acid (FFA) levels were measured in triplicate using an automatic biochemical analyzer (Beckman-700, Fullerton, CA, USA). The aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and γ-glutamyl transpeptidase (γ-GT) enzymes in serum and in liver and kidney tissues were analyzed according to the method in King and Armstrong (1988), and Rosalki and Rau (1972).

2.3.6. Assessment of oxidative stress markers

The supernatants collected after centrifugation of the liver, kidney, and pancreas homogenates were used to determine the GSH and MDA levels. GSH was measured as described in Draper and Hadley (1990) using 5,5 ı-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent), which produces a yellow-colored 5-thio-2-nitrobenzoic acid (TNB). The absorbance of TNB at 412 nm provides an accurate estimation of the level of GSH in a sample. A lipid peroxidation assay was used to determine the amount of MDA, an end product of fatty acid peroxidation that reacts with thiobarbituric acid to form a colored complex with a maximum absorbance at 532 nm, as stated in Shain and Gusmuslu (2007).

2.3.7. Assessment of pro-inflammatory cytokines

Serum and the supernatant collected after centrifugation of the pancreas and kidney homogenates were used for the measurement of TNF-α, IL-1β, and IL-6 levels with a rat TNF-α, IL-1β, and IL-6 ELISA kit (eBioscience, San Diego, CA, USA) according to the manufacturer protocol.

2.4. Statistical analysis

All values are expressed as mean ± standard deviation (SD). The significant differences between the means of the experimental groups was determined with analysis of variance (ANOVA), followed by a Tukey–Kramer multiple comparisons test (Graph Pad version 5.0; Graph Pad Software Inc., San Diego, CA, USA).

3. Results

3.1. Total phenolic and flavonoid contents in CAMFs

The final yield of the obtained CAMFs was 12.6% w/w. The total phenolic and flavonoid contents in CAMFs was determined to be (665.3 ± 188.8 mg GAE/g, and 98.2 ± 27.6 mg Quercetin/g).

3.2. CAMFs analysis by LCMS–MS

The LCMS–MS phytochemical analysis detected quercetin glycoside, 3,4–0-dicaffeoylquinic acid, caffeic acid, naringenin-7-O glucoside and kaempferol as the major compounds in CAMFs, as well as with other unknown compounds (Table 1 and Fig. 1). The fragmentation patterns of known compounds were in agreement with those documented in the literatures as reported in our other study (Arya et al., 2012c).

3.3. Effect of CAMFs on NF-κB activation

We tested CAMFs for its *in vitro* inhibitory effects against H_2O_2-induced NF-κB translocation. NF-κB was detected in the cytoplasm, but not in the nucleus of non-H_2O_2–induced cells. There was a significant increase in NF-κB staining in the nucleus area when cells were stimulated with H_2O_2 alone, suggesting that NF-κB translocated from the cytoplasm into the nucleus. However, treatment with 25 μg/mL CAMFs significantly inhibited H_2O_2-induced NF-κB translocation (Fig. 2).

3.4. Acute toxicity study

No lethality or toxic reactions were observed at any graded doses of CAMFs up to a dosage of 500 mg/kg. Hence, produced no alterations in the blood glucose levels, and on the general behavior or appearance of the rats during the whole experimental period. Based on these findings, the concentration was fixed as 50, 25 and 10 mg/kg bw; which were used as the maximum doses for

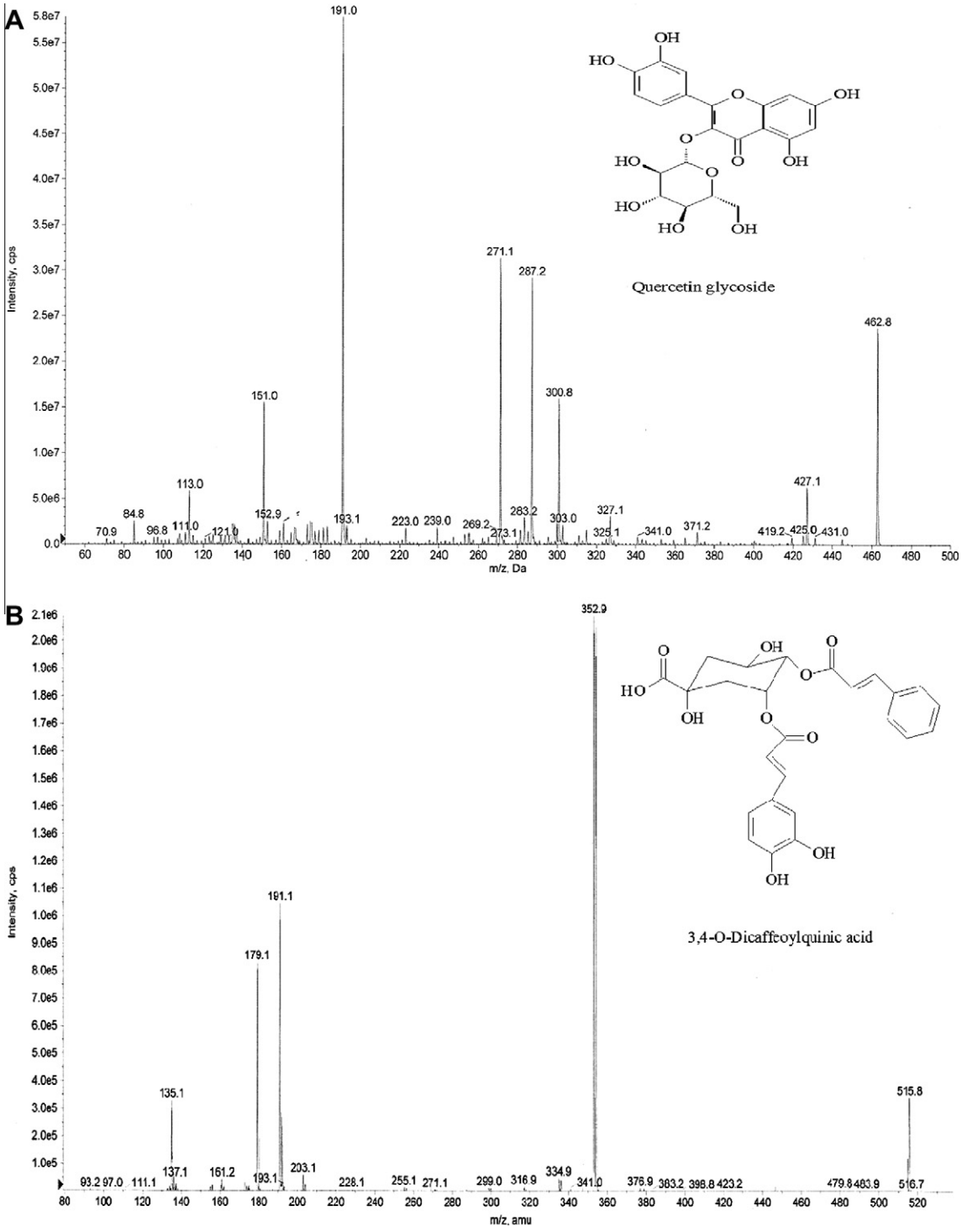


Fig. 1. (A) The mass spectrometric characterization of compound A indicates the presence of quercetin glycosides. (B) The fragmentation pattern of compound B displays an ion peak at *m/z* 515, indicating the loss of the first caffeoyl, loss of the second caffeoyl at *m/z* 353, and loss of the third caffeoyl at *m/z* 191 to yield quinic acid, a loss of caffeic acid at *m/z* 179, and another caffeoyl at *m/z* 135. Thus, the compound was characterized as 3,4–0-dicaffeoylquinic acid. (C) Compound C exhibits fragment ions at *m/z* 179 and *m/z* 135, the characteristic ions of caffeic acid. (D) Compound D was identified as naringenin-7–0-glucoside based on mass fragment ions at *m/z* 433 and at *m/z* 271 and 153. (E) Compound E was identified as kaempferol based on mass fragments at *m/z* 287 and *m/z* 151.

the subsequent experiments involving oral administration as reported in our other study on CAMFs (Arya et al., 2012c).

3.5. Effect of CAMFs on blood glucose levels

From the first day up until the end of the 12-week treatment period, monitoring of the rats' fasting blood glucose levels revealed a significant reduction in the elevated blood glucose levels of diabetic rats treated with CAMFs compared to that of untreated diabetic rats (Table 2). At weeks 3 and 6, the inhibition percentage of blood glucose levels in CAMFs-treated diabetic rats was lower compared to that of glibenclamide-treated diabetic rats. At weeks 9 and 12, the percentage inhibition in rats treated with a higher concentration of CAMFs was greater than that in glibenclamide-treated rats. At the end of the treatment period, the percentage inhibition effected by 50, 25, and 10 mg/kg bw CAMFs was

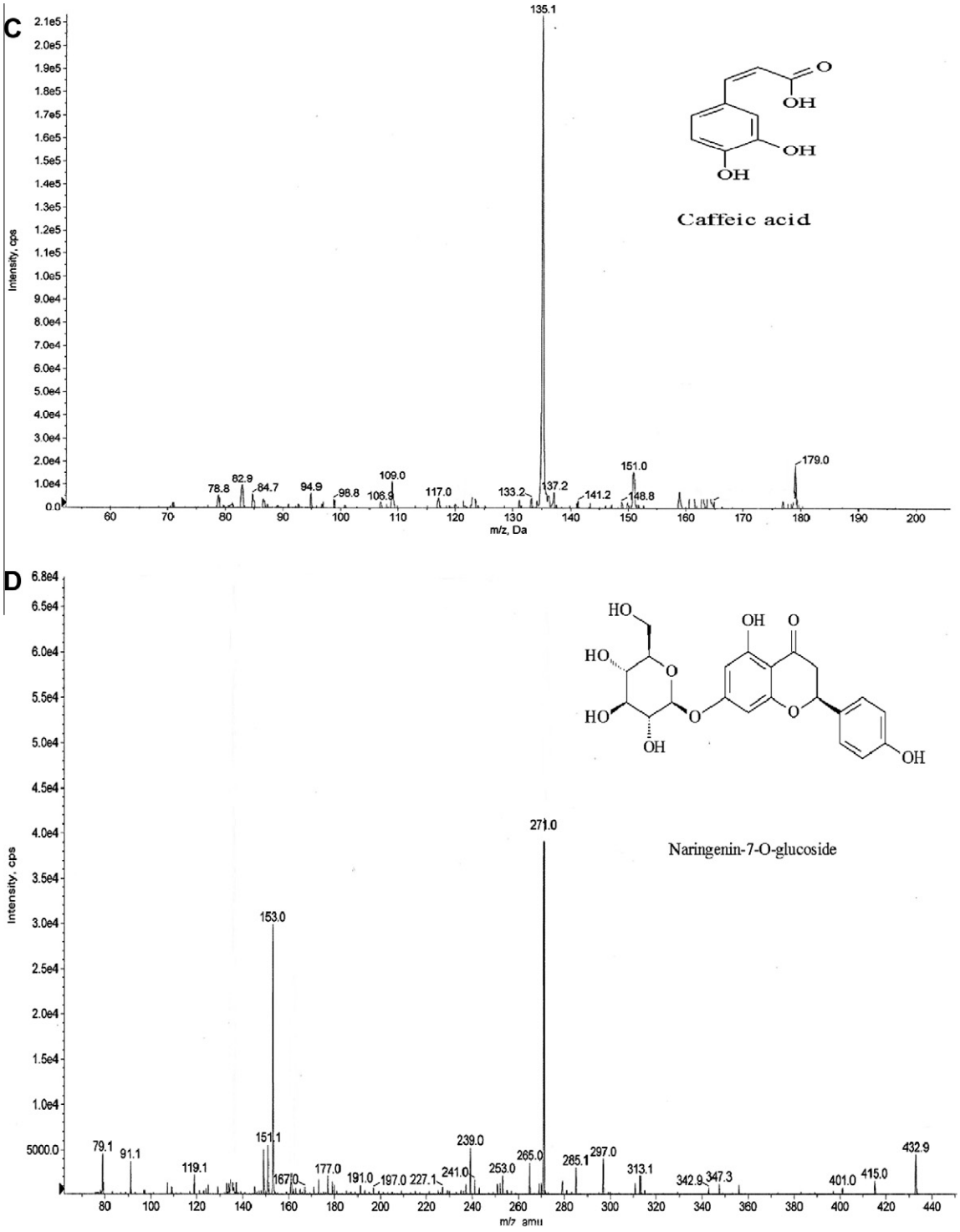


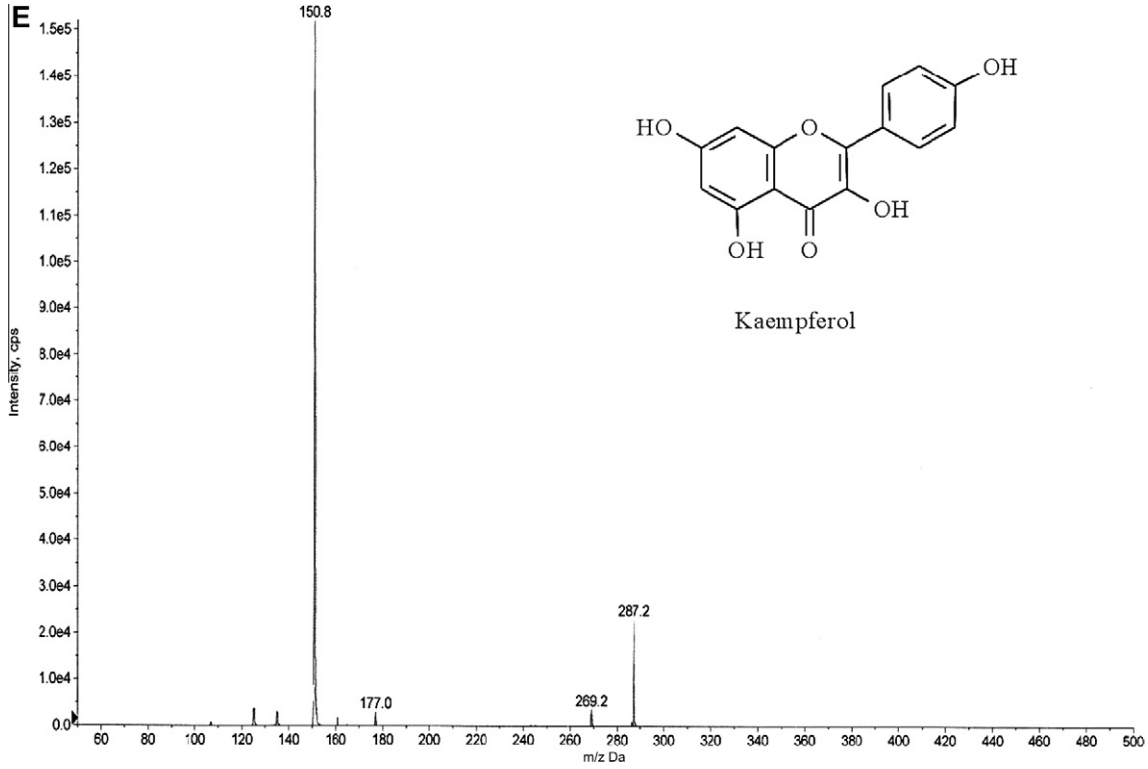
Fig. 1. (*continued*)

75.86%, 69.82%, and 65.08%, respectively, compared to that of glibenclamide (68.53%). The blood glucose levels of normal rats were not greatly altered after week 12 when compared to that of week 0, whereas that of the untreated diabetic rats increased to 23.2 mmol/L after 12 weeks as compared to 12.3 mmol/L at week 0.

3.6. Effect of CAMFs on serum biochemical parameters

After the 12-week treatment period, serum insulin, C-peptide, total protein, and albumin levels in diabetic untreated rats were significantly reduced, while their HbA1c levels were significantly elevated compared to that of the normal control group. Upon treatment with CAMFs or glibenclamide, serum insulin, C-peptide, total protein, and albumin levels in diabetic rats were significantly elevated, and CAMFs significantly inhibited HbA1c levels in a dose-dependent manner compared to that of the diabetic control rats (Fig. 3), indicating significant improvement in glycemic control by CAMFs in diabetic rats.

In addition, there was a significant improvement in the overall body weights of CAMFs-treated diabetic rats compared to untreated diabetic rats at the end of the treatment period, and we recorded weight recovery in the liver and kidney. Whereas



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there was a significant reduction in the daily consumption of food by diabetic treated rats (Fig. 4).

3.7. Effect of CAMFs on hyperlipidemia

Fig. 5 depicts the effect of CAMFs and glibenclamide on serum TG, TC, HDL-C, LDL-C, and FFA activity in treated and untreated diabetic rats and in normal control rats. Serum TG, TC, LDL-C, and FFA levels in untreated diabetic rats were significantly elevated when compared to that of normal control rats, while the HDL-C levels in untreated diabetic rats were significantly decreased compared to those in normal rats.

Serum TG, TC, LDL-C, and FFA levels were significantly reduced after treatment with glibenclamide and CAMFs; CAMFs effected a dose-dependent reduction, and HDL-C levels were significantly elevated compared to that of untreated diabetic rats after the 12-week treatment period. Evidently, continuous treatment with CAMFs decreased these lipid parameters in diabetic rats to near normal levels.

3.8. Effect of CAMFs on enzyme markers

Table 3 illustrates the effects of CAMFs on AST, ALT, ALP, and γ-GT enzyme activities in the serum, liver, and kidney of treated and untreated diabetic rats and in that of normal rats. The enzyme activities in the serum and liver of the untreated diabetic rats were significantly increased; ALP and γ-GT activities in the kidney were increased, while AST and ALT activities were not affected. Near-normalization of the AST, ALT, ALP, and γ-GT activities in the serum, liver, and kidney of diabetic rats was achieved after 12-week administration of glibenclamide and CAMFs; CAMFs effected normalization dose-dependently.

3.9. Effect of CAMFs on oxidative stress markers

GSH levels in untreated diabetic rats were reduced while MDA levels were significantly elevated compared to that of normal control rats (Fig. 6). Compared to untreated diabetic rats, GSH levels in the liver and pancreas of diabetic rats were significantly elevated upon administration of glibenclamide and CAMFs; CAMFs effected elevation dose-dependently, although not much change was observed in kidney GSH levels. There was maximum reduction of MDA levels in the liver of diabetic rats treated with glibenclamide and CAMFs compared to that of untreated diabetic control rats; reduction was effected dose-dependently by CAMFs, while there was a significant reduction of MDA in the pancreas following administration of glibenclamide and 50 mg/kg bw CAMFs. In the kidney, only glibenclamide suppressed MDA levels significantly, with CAMFs effecting a slight reduction in a dose-dependent manner.

3.10. Effect of CAMFs on pro-inflammatory cytokines level

The levels of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 in the pancreas, kidney, and serum of diabetic untreated rats were significantly elevated compared to that of normal control rats (Fig. 7). Compared to untreated diabetic rats, the levels of TNF-α in the pancreas, kidney and serum of diabetic rats were significantly down-regulated by daily administration of glibenclamide and CAMFs after the 12-week period; CAMFs down-regulated these parameters dose-dependently. In the same manner, glibenclamide and CAMFs significantly reduced the IL-1β and IL-6 levels in the pancreas, with non-significant reduction being observed in the kidney and serum after the 12-week study period.

4. Discussion

In an effort to uncover novel and effective treatment from plants traditionally used in India for the management of diabetes mellitus and its associated complications, we evaluated the hypoglycemic effects of a number of plants in an *in vivo* experiment and selected the most potent plant, *C. anthelminticum*, (Arya et al., 2012c) whose seed yields a crude fraction that exerts maximum glycemic control. To the best of our knowledge, this is the first

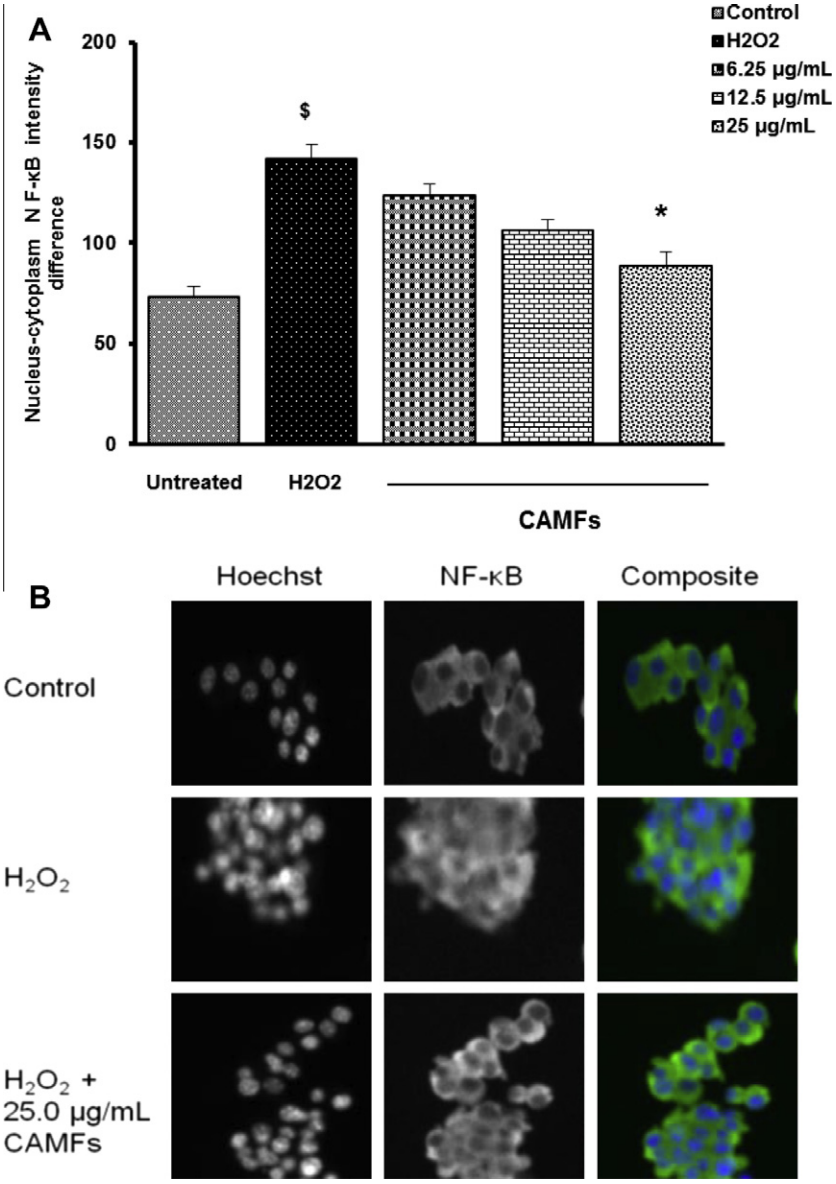

Fig. 2. Stained β-TC6 cells were treated with different concentrations of CAMFs for 1 h and stimulated for 30 min with 50 µM H_2O_2 (NF-κB activation). (A) Dose–response histogram of CAMFs-treated β-TC6 cells for quantitative image analysis of NF-κB translocation. (B) Representative images of control, H_2O_2, and H_2O_2 pre-treated cells treated with 25.0 µg/mL CAMFs. [$]Significant difference compared to non-H_2O_2–treated cells ($P < 0.05$). [*]Significant difference compared to H_2O_2-treated cells ($P < 0.05$).

Table 2
Effects of CAMFs and glibenclamide on blood glucose levels of normal, diabetic control, and diabetic treated rats.

Group	Fasting blood glucose level (mmol/L)				
	Pretreatment period	Treatment period			
	Week 0	Week 3	Week 6	Week 9	Week 12
Normal control	3.9 ± 0.23	3.4 ± 0.26	4.2 ± 0.32	4.1 ± 0.39	4.6 ± 0.37
Diabetic control	12.3 ± 0.44	14.9 ± 0.64	17.8 ± 0.89	19.9 ± 1.09	23.2 ± 2.03
Glibenclamide (50 mg/kg)	12.2 ± 0.65	6.9 ± 0.33[a] (53.69)	6.6 ± 0.48[a] (62.92)	7.0 ± 0.76[a] (64.82)	7.3 ± 0.79[a] (68.53)
CAMFs (50 mg/kg)	11.9 ± 0.53	7.8 ± 0.34[a] (47.65)	6.7 ± 0.32[a] (62.35)	6.2 ± 0.48[a] (68.84)	5.6 ± 0.46[a] (75.86)
CAMFs (25 mg/kg)	12.1 ± 0.46	8.8 ± 0.65[a] (40.93)	7.5 ± 0.47[a] (57.85)	7.1 ± 0.65[a] (64.32)	7.0 ± 0.57[a] (69.82)
CAMFs (10 mg/kg)	12.3 ± 0.51	9.8 ± 0.43[a] (34.22)	8.7 ± 0.77[a] (51.12)	8.9 ± 0.70[a] (55.27)	8.1 ± 0.83[a] (65.08)

Values denote mean ± SD, $n = 9–10$.
Values in parentheses denote that group's percentage decrease in blood glucose level when compared to the diabetic control group in that week.
[a] Mean values that are significantly different from diabetic control group as revealed by the Tukey–Kramer multiple comparisons test ($P < 0.05$).

in vitro cell–based and *in vivo* preclinical study on the crude methanolic fraction of the seed (CAMFs), which demonstrated great therapeutic utility in the management of complications associated with type 2 diabetes.

We evaluated the effects of CAMFs in an animal model of insulin resistance, STZ-nicotinamide–induced DM2, which produces greatly similar features to DM2 in rats (Like and Rossini, 1976; Shima et al., 1998). Daily administration of varying concentrations

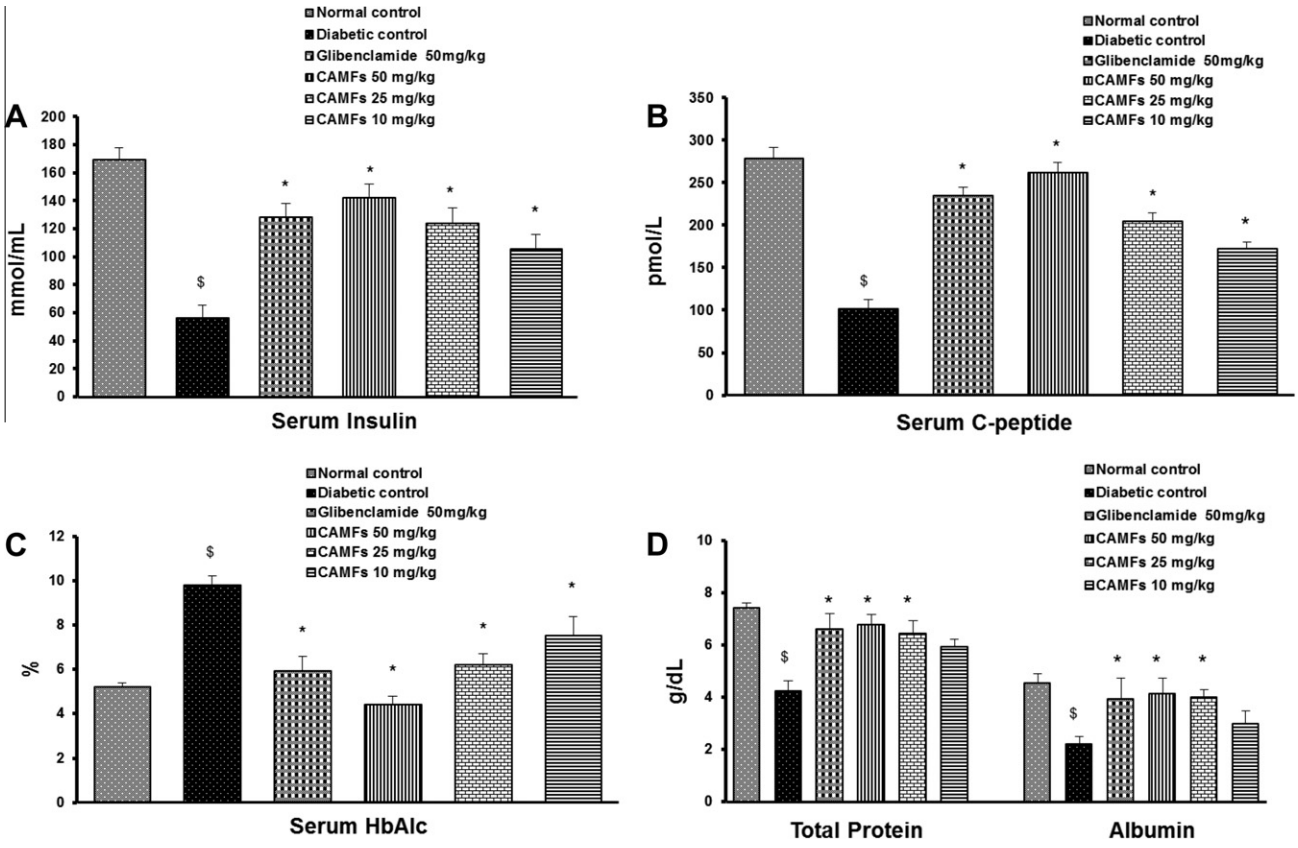

Fig. 3. Effects of CAMFs on serum biochemical parameters of STZ-nicotinamide–induced diabetic rats in comparison with normal and diabetic control rats after the 12-week treatment period. At the end of the treatment, rats were fasted for 12 h and blood was drawn to collect the serum. Panels denote (A) insulin, (B) C-peptide, (C) HbA1c, and (D) total protein and albumin levels. Data are presented as means ± SD (n = 9–10). $^{\$}$Significant difference compared to normal control group ($P < 0.05$). *Significant difference compared to diabetic control ($P < 0.05$).

of CAMFs to diabetic rats for 12 weeks significantly reduced blood glucose levels, thereby increased insulin and C-peptide levels. Elevated insulin levels in diabetics usually normalize the serum and tissue proteins by increasing protein synthesis, decreasing protein degradation or protein glycosylation, supporting our study (Almdal and Vilstrup, 1988). In addition, we observed a reduction in HbA1c level. High HbA1c level has been linked to micro- and macrovascular diabetes complications (Selvin et al., 2004), while decline in HbA1c level reduced morbidity and mortality (Wagner et al., 2001). The recovery of body and organ weights observed in the CAMFs-treated diabetic rats could be due to an improvement in insulin secretion and glycemic control produced by CAMFs. In line with the normalized serum lipid levels, CAMFs restored elevated levels of the enzyme markers AST, ALT, ALP, and γ-GT to normal in the serum, liver, and kidney of diabetic rats. This suggests that CAMFs can be helpful in preventing hepatocellular damage and tissue necrosis through suppression of gluconeogenesis.

Furthermore, we studied the role of ROS in the development of DM2 by activating stress signaling pathways known to participate in insulin signaling pathways, such as the ROS-induced NF-κB activation pathway (Bierhaus et al., 2001; Mohamed et al., 1999; Schreck et al., 1992). Our study result demonstrated that CAMFs inhibited the H_2O_2-induced NF-κB translocation from the cytoplasm into the nucleus in β-TC6 cells (Fig. 2). Moreover, in line with the inhibitory effects of NF-κB translocation, we confirmed antioxidant effects by investigating oxidative stress markers, i.e., GSH and MDA levels in CAMFs treated and untreated diabetic rats, which showed that CAMFs dose-dependently augmented GSH production in the pancreas, kidney, and liver of diabetic rats, with reduction caused in MDA levels, suggesting CAMFs antioxidant ability. It is well established that GSH is an intracellular antioxidant with several biological functions, such as cellular protection against oxidation, which is one of the more important GSH functions because its sulfhydryl (SH) group is a strong nucleophile that confers antioxidant protection and protects DNA, proteins, and other biomolecules from ROS (Fang et al., 2002). In this regard, an increased level of GSH implicates augmentation of the antioxidant capacity and reduced peroxidation of membrane lipids, whose principal end product is MDA, which is a marker of damage caused by oxidative stress (Johansen et al., 2005; Pastore et al., 2003). However, we should not disregard the fact that the antioxidant potential of CAMFs may be, in part, a result of the reduction in hyperglycemia and elevated pro-inflammatory cytokines levels as observed in the diabetic rats.

As we know, DM2 is linked with oxidative stress resulting from free radicals/ROS. ROS act as intercellular second messengers downstream of many signaling molecules, including transcription factors (NF-κB), which mediate vascular smooth muscle cell (SMc) growth/migration and the expression of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (Reuter et al., 2010; Touyz, 2004).These elevated pro-inflammatory cytokines possess antagonistic properties to insulin because of their ability to augment insulin receptor substrate (IRS) phosphorylation, leading to insulin resistance (Emanuelli et al., 2000; Senn et al., 2003; Steinberg, 2007; Tataranni and Ortega, 2005). Therefore, inhibition of H_2O_2-induced NF-κB translocation in β-TC6 cells and ameliorating oxidative stress in diabetic rats explains an associative relationship between the inflammatory cytokines and type 2 diabetes, as shown by our study results on the elevated levels of TNF-α, IL-1β, and IL-6

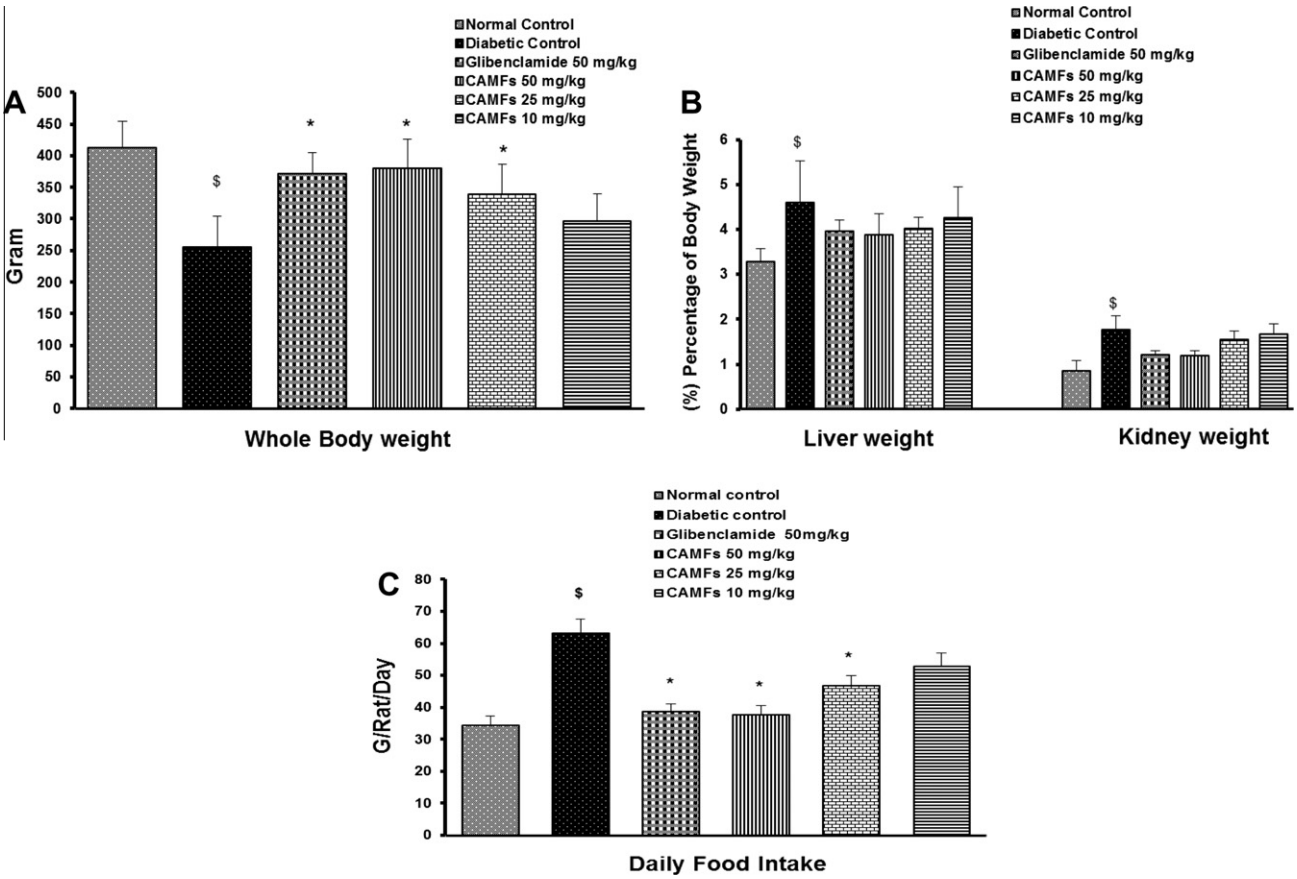

Fig. 4. Effects of CAMFs on (A) whole body weight (B) weight of liver and kidney and (C) daily food intake of STZ-nicotinamide–induced diabetic rats after 12 weeks in comparison with normal and diabetic control rats. Data are presented as means ± SD (n = 9–10). $Significant difference compared to normal control group ($P < 0.05$). *Significant difference compared to diabetic control ($P < 0.05$).

in the serum and tissues of CAMFs treated diabetic rats, suggesting beneficial anti-inflammatory effect of CAMFs on inulin resistance in DM2.

Taken together, our data indicate that 50 mg/kg bw of CAMFs possessed effects that were highly similar to the same dose of glibenclamide. Interestingly, this might be due to the presence of

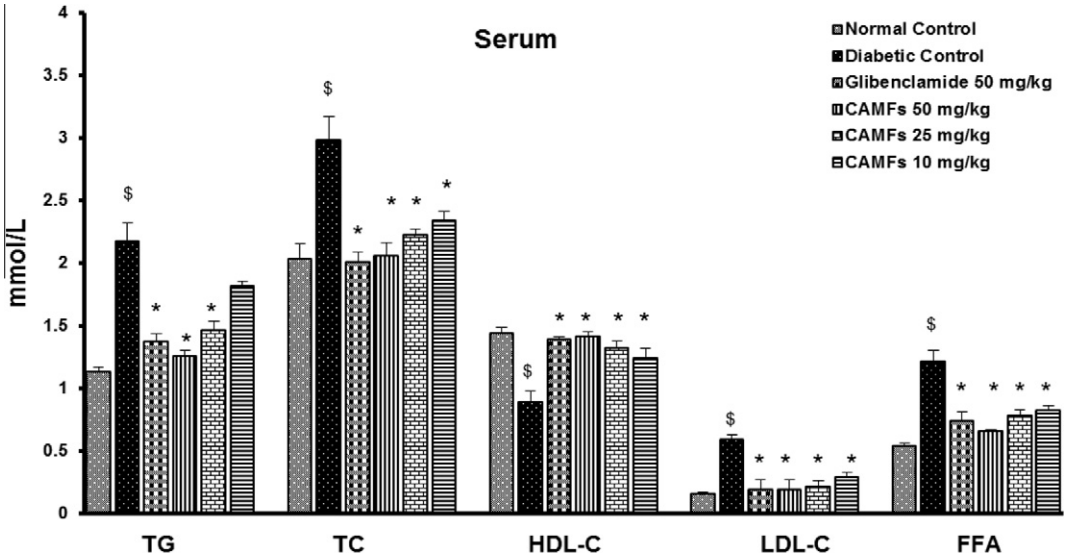

Fig. 5. Effects of CAMFs on serum lipid profiles of STZ-nicotinamide–induced diabetic rats in comparison with normal and diabetic control rats after the 12-week treatment period. At the end of the treatment, rats were fasted for 12 h and blood was drawn to collect the serum. Figure represents TG, TC, HDL-C, LDL-C, and FFA levels in 6 groups. Data are presented as means ± SD (n = 9–10). $Significant difference compared to normal control group ($P < 0.05$). *Significant difference compared to diabetic control ($P < 0.05$).

Table 3
Effect of CAMFs and glibenclamide on enzymes marker in the serum, liver, and kidney of STZ-nicotinamide–induced diabetic rats in comparison with normal and diabetic control rats after the 12-week treatment period.

Group	AST[a]	ALT[b]	ALP[c]	γ-GT[d]
Serum				
Normal control	49.9 ± 3.66[e]	43.7 ± 2.64[e]	67.1 ± 3.14[e]	19.7 ± 1.63[e]
Diabetic control	87.5 ± 3.73	72.2 ± 3.65	107.3 ± 4.49	33.4 ± 1.05
Glibenclamide (50 mg/kg)	55.4 ± 2.92[e]	48.6 ± 2.03[e]	69.4 ± 4.23[e]	21.1 ± 1.01[e]
CAMFs (50 mg/kg)	52.3 ± 2.53[e]	44.2 ± 1.36[e]	57.6 ± 2.07[e]	19.8 ± 1.75[e]
CAMFs (25 mg/kg)	62.2 ± 3.23[e]	57.8 ± 2.02[e]	77.4 ± 5.32[e]	25.7 ± 1.26
CAMFs (10 mg/kg)	73.7 ± 3.12	63.7 ± 2.47	81.2 ± 6.41[e]	30.1 ± 2.73
Liver				
Normal control	723.7 ± 13.6[e]	883.8 ± 17.25[e]	0.19 ± 0.52[e]	3.11 ± 0.26[e]
Diabetic control	937.2 ± 20.18	1259.6 ± 26.31	0.36 ± 0.21	5.53 ± 1.21
Glibenclamide (50 mg/kg)	754.3 ± 17.38[e]	905.2 ± 16.42[e]	0.22 ± 0.23	3.31 ± 0.87
CAMFs (50 mg/kg)	757.5 ± 18.23[e]	876.8 ± 22.43[e]	0.20 ± 0.13	3.01 ± 0.13[e]
CAMFs (25 mg/kg)	807.2 ± 19.36	923.4 ± 21.41	0.29 ± 0.76	3.82 ± 0.17
CAMFs (10 mg/kg)	893.4 ± 22.46	1033.5 ± 21.32	0.33 ± 0.26	4.23 ± 0.72
Kidney				
Normal control	848.2 ± 19.31	935.7 ± 21.67	0.43 ± 0.16[e]	2.96 ± 0.87[e]
Diabetic control	839.4 ± 18.36	929.4 ± 21.61	0.98 ± 0.56	4.21 ± 0.74
Glibenclamide (50 mg/kg)	852.4 ± 24.26	927.3 ± 24.18	0.50 ± 0.32[e]	3.02 ± 0.21
CAMFs (50 mg/kg)	846.3 ± 22.43	944.5 ± 20.78	0.39 ± 0.13[e]	2.79 ± 0.32[e]
CAMFs (25 mg/kg)	826.1 ± 18.21	903.2 ± 20.13	0.58 ± 0.34	3.16 ± 0.56
CAMFs (10 mg/kg)	818.9 ± 21.23	896.5 ± 22.79	0.68 ± 0.23	3.53 ± 0.19

Values denote mean ± SD, $n = 9–10$.
Units of measurement (per L) for AST and ALT: μmol of pyruvate liberated/h; ALP: μmol of phenol liberated/min; γ-GT: mol of p-nitroaniline liberated/min.
[a] Aspartate transaminase.
[b] Alanine transaminase.
[c] Alkaline phosphatase.
[d] γ-Glutamyltranspeptidase.
[e] Mean values that are significantly different from diabetic control in the same group, as revealed by the Tukey–Kramer multiple comparisons test ($P < 0.05$).

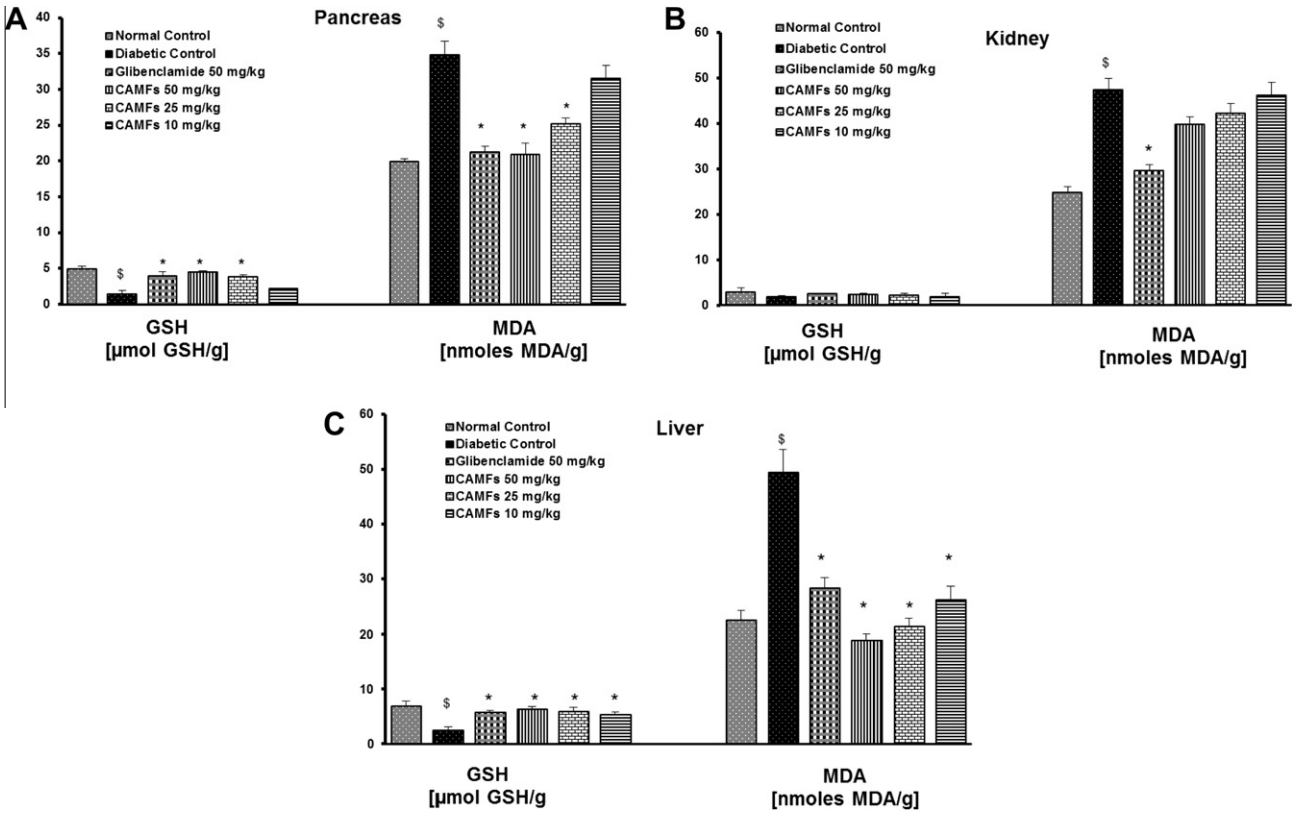

Fig. 6. Antioxidant effect of CAMFs on the pancreas, kidney, and liver of STZ-nicotinamide–induced diabetic rats in comparison with normal and diabetic control rats after the 12-week treatment period. At the end of the treatment, the organs were removed, homogenized, and centrifuged to collect the tissue supernatant. Panels denote (A) pancreas, (B) kidney, and (C) liver, GSH and MDA levels respectively. Data are presented as means ± SD ($n = 9–10$). $^{\$}$Significant difference compared to the normal control group ($P < 0.05$). *Significant difference compared to the diabetic control ($P < 0.05$).

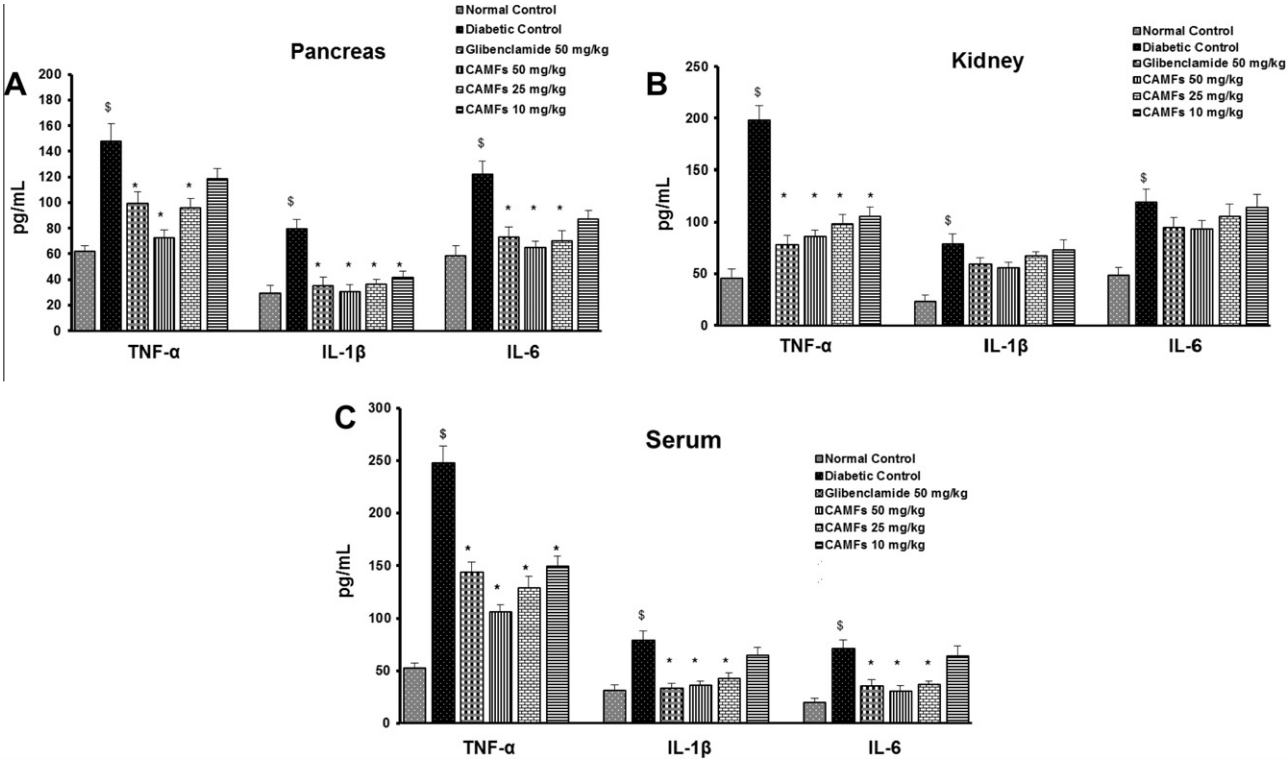

Fig. 7. Anti-inflammatory effect of CAMFs and glibenclamide in STZ-nicotinamide–induced diabetic rats in comparison with normal and diabetic control rats after the 12-week treatment period. At the end of the treatment, the blood and organs were removed and centrifuged to collect serum from the blood and tissue supernatant from the homogenized organs. Panels denote (A) pancreas, (B) kidney, and (C) serum, respectively, illustrating TNF-α, IL-1β, and IL-6 levels. Data are presented as means ± SD (n = 9–10). $^{\$}$Significant difference compared to normal control group ($P < 0.05$). *Significant difference compared to the diabetic control ($P < 0.05$).

the phytochemicals in CAMFs, such as quercetin glycoside, 3,4–0-dicaffeoylquinic acid, caffeic acid, naringenin-7–0-glucoside and kaempferol. Several studies on these compounds have demonstrated antidiabetic, antioxidant and anti-inflammatory properties (Jung et al., 2006; Ortiz-Andrade et al., 2008; Shih et al., 2012). Thus, the combination of these compounds in CAMFs may be responsible for the synergistic effects observed in STZ-nicotinamide induced type 2 diabetic rats.

5. Conclusion

The crude methanolic fraction of *C. anthelminticum* seeds (CAMFs) displayed inhibitory effects on NF-κB translocation in H_2O_2-stimulated β-TC6 cells. This observation was corroborated with the beneficial effects of CAMFs in attenuating hyperglycemia, by down-regulating elevated levels of pro-inflammatory cytokines, oxidative stress and hyperlipidemia in an animal model of type 2 diabetes. These findings pave the way for a novel approach to potential treatments of insulin-resistant type 2 diabetes and propose CAMFs as a valuable candidate nutraceutical for the type 2 diabetic complications.

Conflict of Interest

The authors disclose no conflicts of interest.

Role of the funding source

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3.3.4.1 Histopathological study (unpublished data)

At the end of 12-week study period, all the experimental animals (SD rats): normal control, STZ-nicotinamide-induced diabetic and CAMFs treated diabetic rats were fasted for 12 hours and blood was collected for serum analysis, thereafter all the group animals were sacrificed by cervical dislocation. The liver, kidney and pancreas were removed, washed in ice-cold isotonic saline, and blotted individually on ash-free filter paper; the organs were weighed and cut into pieces for the estimation of markers as well as for histology. For histology, organs were fixed in 10% buffered formalin for 48 hours, after that rinsed with xylene and embedded in paraffin. Thereafter, (5 μm thick) sections were prepared and stained with hematoxylin and eosin dye (H&E). Finally, all the sections were mounted using neutral deparaffinated xylene (DPX) medium for microscopic examination on a Motic BA 400 microscope using Motic Advance 3.0 software.

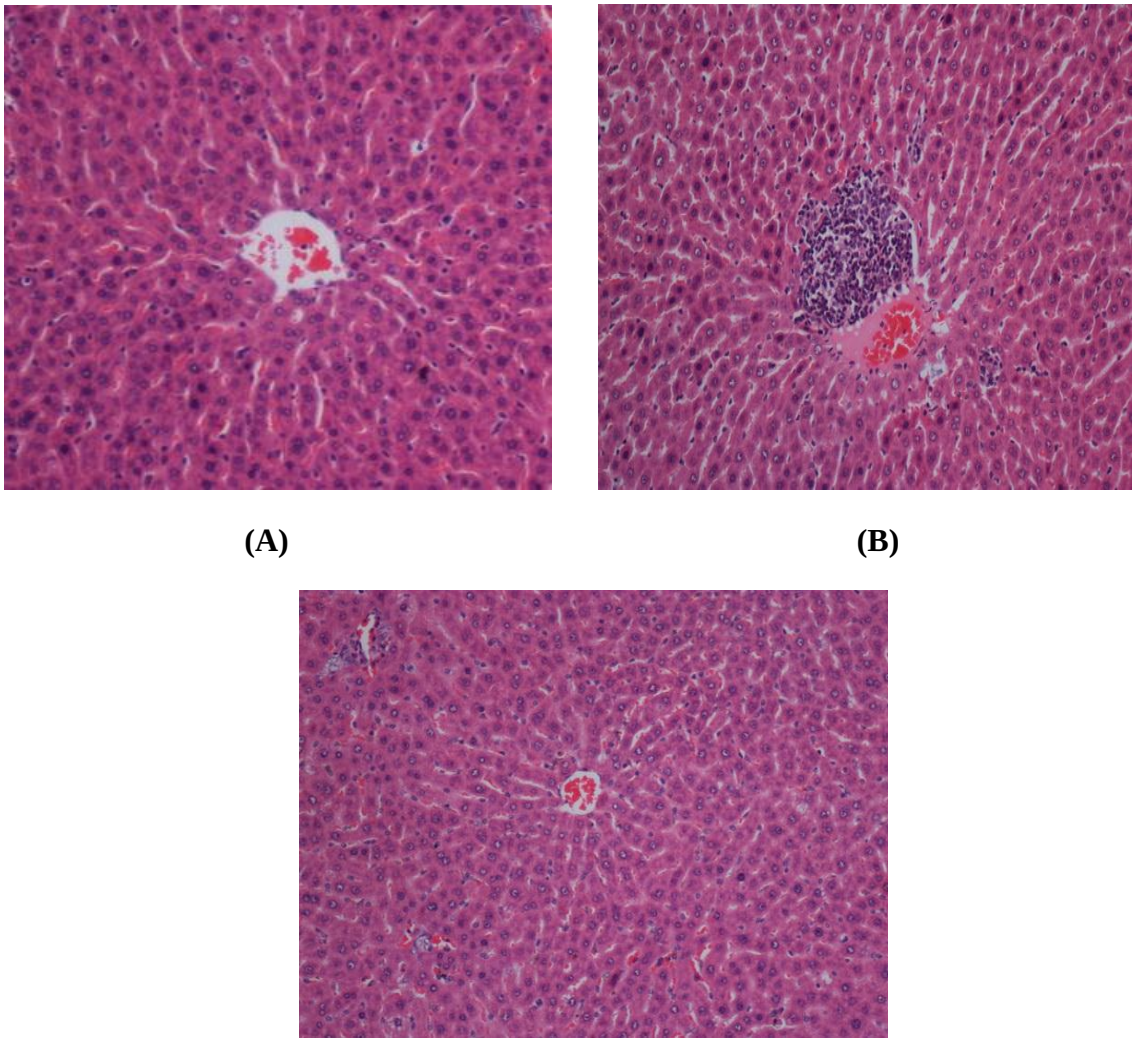
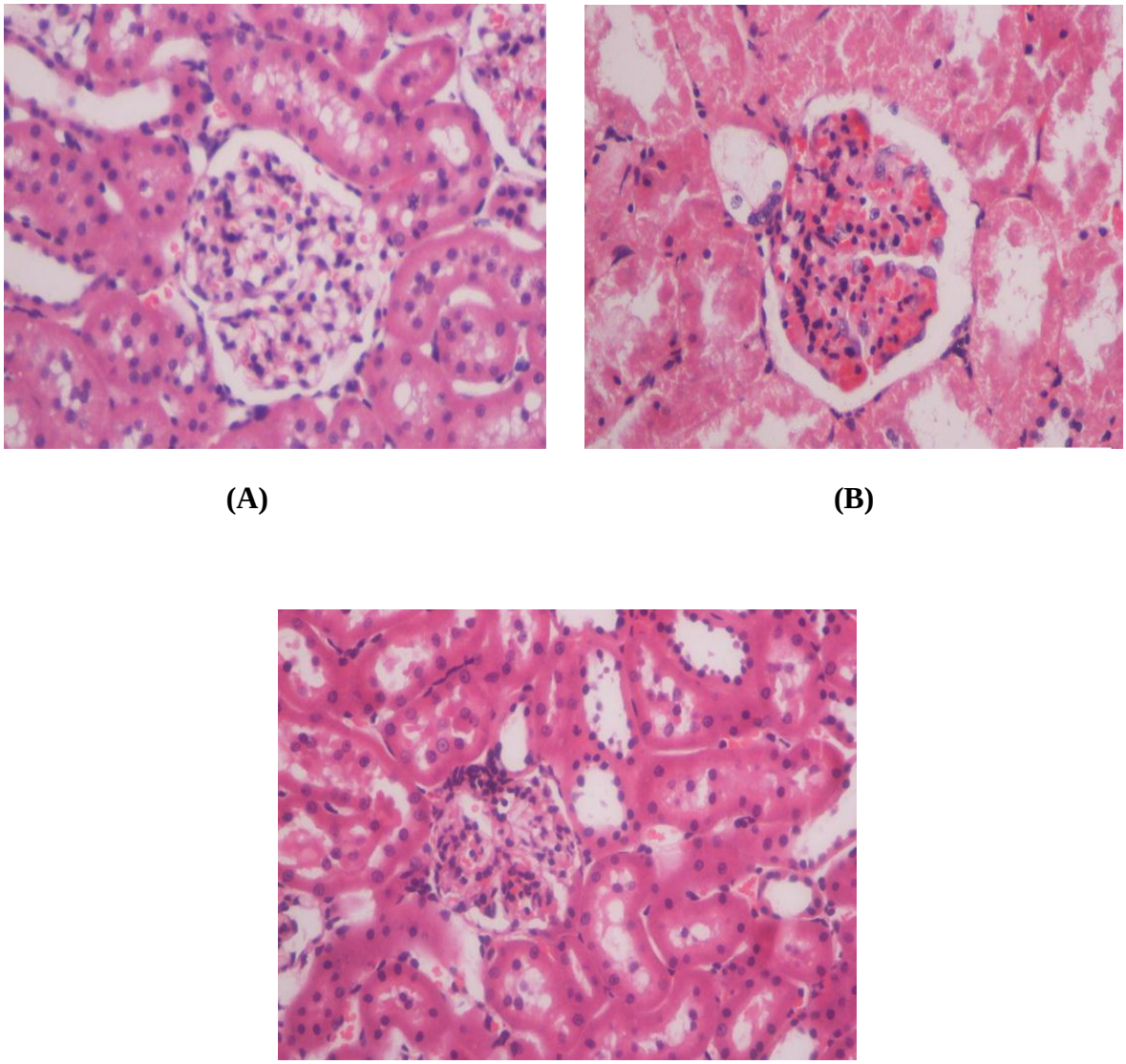


Figure 3.2: Histology of liver

Photomicrographs (40xx) displaying liver of normal control rats, diabetic control and diabetic rats treated with CAMFs. (A) Liver of normal rats showed normal hepatic structure without any abnormallity. (B) Liver of STZ-nicotinamide-induced diabetic rats showed disorganized hepatic cords, reduced sinusoids with dilation, feathery degeneration and many hepatocytes having cytoplasmic vacuolar degeneration, pyknotic nuclei and central inflammatory cell infiltration with abscess formation. (C) CAMFs (50 mg/kg) treated diabetic rats prevented cytoplasmic vacuolar degeneration of hepatocytes and recovered accumulated inflammatory cell infiltration, with fewer pyknotic nuclei, compared to diabetic control rats.

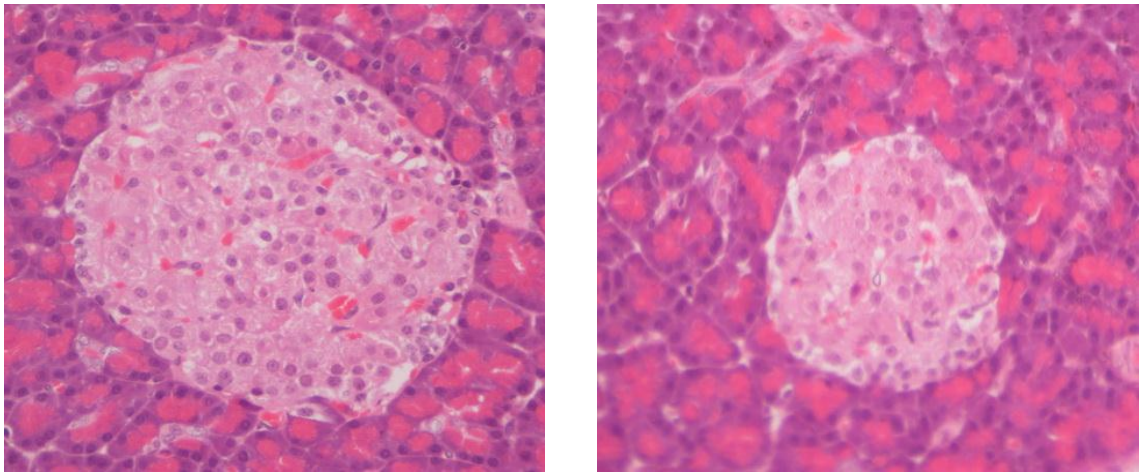


(A) **(B)**

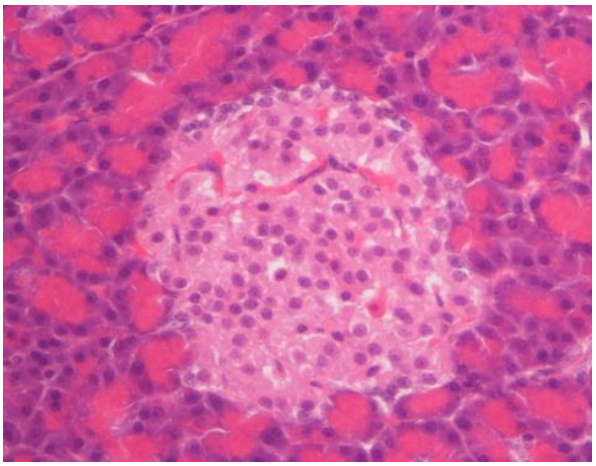
(C)

Figure 3.3: Histology of kidney

Photomicrographs (40xx) displaying kidney of normal control rats, diabetic control and diabetic rats treated with CAMFs. (A) Kidney of normal rats, showed normal parenchymal structure of kidney. (B) Kidney of STZ-nicotinamide-induced diabetic rats showed severe tubular epithelial atrophy with mesangial proliferation, abnormal sclerotic changes in the glomerulus, as well as congestion in the capillaries with infiltaration of mononuclear cells in renal parenchyma. (C) Diabetic rats treated with CAMFs (50 mg/kg), demonstrated mild tubular epithelial atrophy with less congestion in capillaries and recovery of inflammed mononuclear cells in renal parenchyma.



(A) **(B)**



(C)

Figure 3.4: Histology of pancreas

Photomicrographs (40xx) displaying pancreas of normal control rats, diabetic control and diabetic rats treated with CAMFs. (A) Pancreas of normal rats, exhibited normal pancreatic structure with intact alpha, beta and delta islets cells of Langerhans. (B) Pancreas of STZ-nicotinamide-induced diabetic rats showed marked decrease in number of constituting islet cells with hyperplasia and exhibiting necrosis, vacuolations, hydropic cells with pyknotic nuclei, and also congestion in pancreatic parenchyma with infiltration of cells. (C) Diabetic rats treated with CAMFs (50 mg/kg) increased number of constituting islet cells and reduced necrosis, vacuolations, hydropic cells and pyknotic nuclei with recovery in the infiltration of inflammatory cells.

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Research Article

Chloroform Fraction of *Centratherum anthelminticum* (L.) Seed Inhibits Tumor Necrosis Factor Alpha and Exhibits Pleotropic Bioactivities: Inhibitory Role in Human Tumor Cells

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We investigated the antioxidant potential, cytotoxic effect, and TNF-α inhibition activity with NF-κB activation response in a chloroform fraction of *Centratherum anthelminticum* seeds (CACF). The antioxidant property of CACF was evaluated with DPPH, ORAC, and FRAP assays, which demonstrated significant antioxidant activity. The cytotoxicity of CACF was tested using the MTT assay; CACF effective inhibitory concentrations (IC_{50}) for A549, PC-3, MCF-7, and WRL-68 cells were 31.42 ± 5.4, 22.61 ± 1.7, 8.1 ± 0.9, and 54.93 ± 8.3 μg/mL, respectively. CACF effectively and dose-dependently inhibited TNF-α release, *in vitro* and *in vivo*. CACF inhibited TNF-α secretion in stimulated RAW264.7 macrophage supernatants with an IC_{50} of 0.012 μg/mL, without affecting their viability; the highest dose tested reduced serum TNF-α by 61%. Acute toxicity testing in rats revealed that CACF was nontoxic at all doses tested. Matching the cytotoxic activity towards a mechanistic approach, CACF dose-dependently exhibited *in vitro* inhibitory effects against the activation of NF-κB translocation in MCF-7 cells. Preliminary phytochemical screening with GC/MS analysis detected 22 compounds in CACF, of which morpholinoethyl isothiocyanate was the most abundant (29.04%). The study reveals the potential of CACF in the treatment of breast cancer and in oxidative stress conditions with associated inflammatory responses.

1. Introduction

Since time immemorial, traditional herbs have been used as remedies for several diseases [1]. Even though new synthetic drugs are available, traditional medicine is still being utilized today as part of primary healthcare in several parts of the world. Traditional medicine is often sought because it is economical, readily available, and trusted by its advocate [2]. Studies on herbal medicines have shown the positive correlation of traditional claims and scientific data [3]. Some of these traditional treatments are part of clinical practice, and few are found to be not beneficial [4]. Support for traditional medicine and the numerous natural products with biological activity have led to multidisciplinary investigations utilizing preliminary screening procedures as well as advanced mechanistic studies to develop drugs which may be used clinically.

Centratherum anthelminticum (L.) KUNTZE (family: Asteraceae) is an erect, pubescent annual herb found widely in the Indian subcontinent which is locally known as "Somraj," and its seeds are known as "Kalijiri" in Hindi [5, 6]; scientific synonyms for this plant include *Vernonia anthelmintica* and *Conyza anthelmintica*, among others. This plant is used extensively in Ayurveda for the treatment of cough and diarrhoea, as well as an anthelmintic, stomachic, diuretic, and antiphlegmatic agent [5, 7]. Experimental studies proved the pharmacological effects of this plant's seeds extract, including antihelminthic [8], larvicidal [9], antipyretic [10], antifilarial [11], antihyperglycemic [12], antimicrobial [13], and diuretic [14] activities. In addition

to primary metabolites, the seeds of this plant contain glycosides, phenolic compounds, tannins, flavonoids, saponins, and sterols [15]. Examples of secondary metabolites found in *C. anthelminticum* include the following: flavonoids such as 2′,3,4,4-tetrahydroxychalcone (Butein); 5,6,7,4′-tetrahydroxy flavone and 7,3′,4′-trihydroxydihydroflavone [16]; sterols such as sterol-4-alpha-methylvernosterol, vernosterol, and avernosterol [17]; steroids such as (24a/R)-stigmasta-7-en-3-one, 24(a/R)-stigmasta-7, 9(11)-dien-3-one, 24(a/S)-stigmasta-5 and 22-dien-3β-ol, stigmasta-7, and 22-dien-3β-ol [18].

While several studies investigated the pharmacological effects of this plant, the antiinflammatory and anti-cancer activities are yet to be investigated. Inflammation is a natural defense mechanism by the host which plays a role in various diseases. Immune cells are particularly important in inflammation because they orchestrate the release of several mediators such as cytokines, prostaglandins, and nitric oxide, which play a part in the defense process. However, uncontrolled production of these mediators is associated with tissue damage due to oxidative stress [19]. Oxidative stress resulting from reactive oxygen and nitrogen species (ROS and RNS) was shown to cause DNA mutations and cell death and affect cell proliferation. Cells which survive the DNA damage caused by oxidative stress are likely to have aberrant repair mechanisms, the proliferation of these genetically instable cells might eventually progress toward carcinogenesis. Therefore, this study looked into the antioxidant approaches, the *in vitro* and *in vivo* TNF inhibition activity counting cytotoxic effect in the chloroform fraction with further evaluation of linkage between NF-κB activation on breast cancer (MCF-7) cell lines.

2. Materials and Methods

2.1. Cell Lines and Reagents. All cell lines were purchased from ATCC (Rockville, MD, USA). RPMI medium, penicillin and streptomycin solution, and phosphate buffer Saline (PBS) were purchased from Invitrogen (Rockville, MD, USA). MTT, DMSO, and heat-inactivated fetal bovine serum and 0.25% trypsin solution were purchased from Sigma-Aldrich Chemicals (Saint Louis, MO, USA). Cell culture treated 96-well plates, and cell culture flasks were purchased from Orange Scientific (Braine-l'Alleud, Belgium). PBS 75 nM, pH Dulbecco's Modified Eagle Medium (DMEM), phosphate buffered saline, Hanks' balanced salt solution (HBSS), and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Invitrogen (Carlsbad, USA). Fetal bovine serum (FBS), LPS from *E. coli* serotype 0111:B4, and dimethylsulfoxide (DMSO) were obtained from Sigma (St. Louis, USA). Murine TNF-α ELISA kit was from eBioscience (San Diego, USA). NF-κB activation kit from Thermo Scientific Cellomics, fluorescence sodium salt, DPPH and FRAP reagents, Pentoxifylline and Paclitaxel (Sigma-Aldrich), AAPH, Quercetin, and Trolox were from Sigma-Aldrich Chemicals (S. Louis, MO, USA). Plates were read using Chameleon V Multilabel microplate reader (Hidex, Turku; Finland) in 96-well format black plate.

2.2. Plant Materials. The seeds of the plant *Centratherum anthelminticum* were procured from the medicinal plant cultivation zone of Amritum Bio-Botanica Herbs Research Laboratory Pvt. Ltd, Betul, Madhya Pradesh, India. The seeds of the plant were authenticated by the Quality Control department of the company itself. Voucher specimen (CA-9) is deposited in the Pharmacology Department of the University of Malaya, Malaysia.

2.3. Extraction Procedure. The coarsely powdered seeds (100 g) were extracted with water : ethanol (80 : 20) using a Soxhlet extractor for 24 h (Figure 1). The solvent was completely evaporated using rotary evaporator. The brown viscous crude extract weighing (49% w/w) obtained was further fractionated successively with hexane, chloroform and methanol, then the solvent from each fraction was completely recovered with the help of rotary evaporator under reduced pressure. After drying, the final yields with hexane (CAHF), chloroform (CACF), and methanol fraction (CAMF) were (19.41% w/w), (4.11 w/w), and (11.3% w/w), respectively. Then the dried fractions were kept below -20°C before being used. The in pilot bioactivity testing, chloroform fraction (CACF) had shown the positive effect and hence was chosen for further analysis.

2.4. Animals. Altogether 72 Sprague Dawley rats of 150–200 g were obtained from the University of Malaya Medical Centre Animal House and maintained under standard conditions of lighting (12 h of light and darkness) and nutrition (food and water *ad libitum*) throughout the experimental period. Studies were performed in accordance with the Medical Research Council Guidelines on Ethics in Animal Experimentation. This study was approved by the Animal Experimentation ethics committee at the University of Malaya Medical Center-(UMMC), Animal Ethics no: FAR/10/11/2008/AA(R).

2.5. Total Phenolic Content (TPC). TPC of CACF was determined using Folin-Ciocalteu method [20]. CACF was prepared in a concentration of 10 mg/mL in methanol. Five microliters of this solution were transferred to 96-well mircoplate (TPP, USA). To this, 80 μL of Folin-Ciocalteu reagent (1 : 10) was added and mixed thoroughly. After 5 min, 160 μL of sodium bicarbonate solution ($NaHCO_3$ 7.5%) was added, and the mixture was allowed to stand for 30 min with intermittent shaking. Absorbance was measured at 765 nm using microplate reader (Molecular Devices, Sunnyvale, USA). The TPC was expressed as gallic acid equivalent (GAE) in mg/g extract and obtained from the standard curve of gallic acid. The gallic acid standard curve was established by plotting concentration (mg/mL) versus absorbance (nm) ($y = 0.001x + 0.045$; $R^2 = 0.9975$), where y is absorbance and x is concentration in GAE ($n = 3$).

2.6. Antioxidant Activity of C. anthelminticum

2.6.1. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity of CACF. The scavenging activity of CACF on DPPH was determined using the method described in

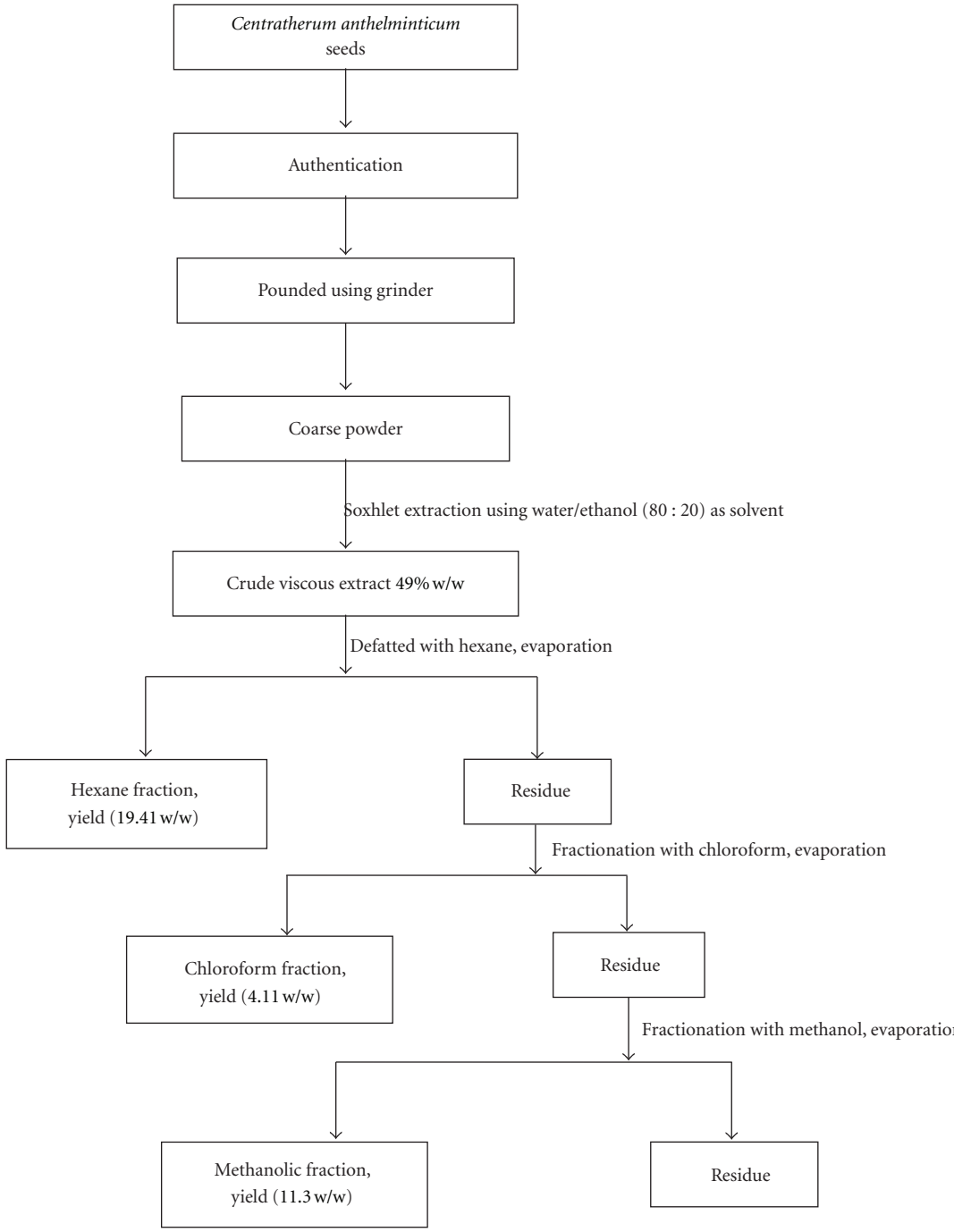

Figure 1: Schematic diagram of extraction/fractionation of *C. anthelminticum* seeds.

[21]. This method is based on the reduction of purple DPPH to a yellow-colored diphenyl picrylhydrazine. Changes in color were measured at 518 nm. CACF was tested at final concentrations ranging from 600 to 10 μg/mL in ethanol. One milliliter of 0.3 mM DPPH ethanol solution was added to 2.5 mL of sample solution of different concentrations to make the test solutions, while 1 mL of ethanol was added to 2.5 mL of samples to make the blank solutions. The negative control (blank) consisted of 1 mL DPPH solution plus 2.5 mL of ethanol. These solutions were allowed to react at room temperature for 30 minutes in the dark. The absorbance values were measured at 518 nm and converted into percentage antioxidant activity using the following equation:

$$\% \text{ Inhibition} = \left[\frac{(A_B - A_A)}{A_B}\right] \times 100, \quad (1)$$

where A_B is the absorption of blank sample; A_A is the absorption of tested samples.

The IC_{50} as well as the kinetics of DPPH scavenging activity was determined. Ascorbic acid and butylated hydroxytoluene (BHT) was used as a positive control in this assay.

2.6.2. ORAC Antioxidant Activity Assay. The oxygen radical absorbance capacity (ORAC) assay was done based on the procedure described earlier by Choi et al. [21] with slight modifications. Briefly, 175 μL of the sample/blank were dissolved with PBS at concentrations of 160 μg/mL, pH 7.4. Serial dilutions of the standard Trolox were prepared from 75 mM. The assay was performed in 96-well black microplates 25 μL of samples (CACF), standard (Trolox), blank (solvent/PBS), or the positive control (quercetin) was added to the wells. Subsequently, 150 μL of fluorescent sodium salt solution was added, and the plate was then incubated for 45 minutes at 37°C. Twenty five microliters of 2,20-azobis (2-amidinopropane) dihydrochloride (AAPH) solution was added for a total volume of 200 μL/well. Fluorescence was recorded until it reached zero (excitation at 485 nm, emission at 535 nm) using a fluorescence spectrophotometer (Perkin—Elmer LS 55), equipped with an automatic thermostatic autocell holder at 37°C. Data were collected every 2 mins for a duration of 2 hrs and were analyzed by calculating the differences of areas under the fluorescein decay curve (AUC) between the blank and the sample. Values were expressed as Trolox equivalents.

2.6.3. Ferric Reducing/Antioxidant Power (FRAP) Assay. The FRAP assay was slightly modified from the method of Benzie and Strain [22]. The stock solutions included 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ, and 2.5 mL $FeCl_3 \cdot 6H2O$. The temperature of the solution was raised to 37°C before use. CACF (10 μL) was allowed to react with 190 μL of the FRAP solution for 30 min in the dark. Colorimetric readings of the product ferrous tripyridyltriazine complex were taken at 593 nm. The standard curve was linear between 200 and 1000 μM $FeSO_4$. Results are expressed as μM Fe (II)/g dry mass and compared with those of Ascorbic acid and BHT.

2.7. Cell Viability and Cytotoxicity

2.7.1. Cell Culture. All cell lines were obtained from American Type Cell Collection (ATCC) and maintained in a 37°C incubator with 5% CO_2 saturation. Human breast carcinoma (MCF-7) and normal hepatic (WRL-68) cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM), while nonsmall cell lung cancer cells (A549) and prostate adenocarcinoma cells (PC-3) were maintained in RPMI medium. Both media were supplemented with 10% fetus calf serum (FCS), 100 units/mL penicillin, and 0.1 mg/mL streptomycin. Cells were cultured using standard aseptic techniques and were seeded at the indicated densities below.

2.7.2. Cellular Viability. The inhibitory effects of CACF on the indicated cell lines' growth were tested using the MTT assay [23]. Cells were seeded at a density of 1×10^5cells/mL in a 96-well plate and incubated for 24 hours at 37°C and 5% CO_2. Cells were then treated with CACF and incubated for another 24 hours, after which the MTT solution at 2 mg/mL was added for 1 hour. The insoluble formazan product was dissolved in DMSO, and absorbance was measured at 570 nm using Plate Chameleon V microplate reader (Hidex, Turku, Finland). Results were expressed as a percentage calculated from the ratio of absorbance of treated cells to untreated cells. The concentration that caused a 50% loss of cell growth (IC_{50}) was used to measure the CACF growth inhibition potency.

2.8. TNF-α Inhibition Activity of CACF

2.8.1. In Vitro Cell Viability and TNF-α Production Assays. Murine macrophage cells RAW 264.7 were seeded in 96-well plates at 5×10^5cells/mL. Cells were either left untreated in DMEM or pretreated with CACF at the indicated concentrations for 30 minutes. Cell stimulation, viability, and TNF production measurements were conducted exactly as in [24]. Percentage viability was calculated as follows: cell viability (%) = $[(OD_{570}\ (\text{sample})/OD_{570}\ (\text{control})) \times 100]$.

Whereas percentage TNF inhibition was calculated as follows:

$$\%\ \text{inhibition} = 100 \times \left[\frac{([\text{TNF}]\ \text{control} - [\text{TNF}]\ \text{sample})}{[\text{TNF}]\ \text{control}}\right], \quad (2)$$

where control indicates cells treated in LPS alone.

2.8.2. Measurement of In Vivo Serum TNF. Healthy male rats were selected and divided into 6 groups ($n = 8$ for each group). The rats were pretreated with the indicated solutions for 30 minutes before lipopolysaccharide (LPS) stimulation: group 1 was pretreated intraperitoneally (i. p.) with 1 mL of PBS alone, group 2 with dexamethasone (6 mg/kg BW) in 25% DMSO, group 3 with CACF (25 mg/kg BW) in 25% DMSO, group 4 with CACF (50 mg/kg BW) in 25% DMSO, and group 5 with CACF (100 mg/kg BW) in 25% DMSO. Groups 1–5 were treatment groups stimulated with LPS, while group 6 made up the untreated negative control in which rats were given a solution of 25% DMSO in PBS and no LPS. Subsequent to pretreatment, LPS (1 mg/kg) was then administered in 1 mL of pyrogen-free normal saline i. p for five treatment groups, and PBS was administered i. p. for the negative control group. Blood was withdrawn from the animals under ether anesthesia after 90 minutes of LPS or PBS administration. Serum was collected and stored at −80°C until analysis. Serum levels of TNF-α were determined using rat TNF-α ELISA kit according to the manufacturer's protocol (e Bioscience, San Diego, USA).

2.9. Acute Toxicity Study. Healthy adult rats of either sex were divided into 4 groups ($n = 6$) and were orally fed with increasing doses of CACF: 10, 20, 100, and 1000 mg/kg body weight (BW). The rats were observed continuously for 2 hours for behavioral, neurological, and autonomic profiles

Table 1: Antioxidant activity profile of the chloroform fraction of *Centratherum anthelminticum* (CACF).

Samples	DPPH IC_{50}, μg/mL	FRAP (μmol/L)	ORAC Equivalent conc. Trolox (20 μg/mL (μM)** IC_{50}		TPC μg GAE/mg
CACF	$22.56^{a} \pm 1.4$	$1048.3^{a} \pm 21.2$	$992.34^{a} \pm 45.12$	86.62	37.16 ± 0.85
Ascorbic acid	$15^{a} \pm 0.3$	$6240^{b} \pm 56.2$	—	—	—
BHT	$17^{a} \pm 0.4$	$907.7^{a} \pm 54.8$	—	—	—
Quercetin	—	—	$1018.00^{b} \pm 34.82$	74.52	—

*The net AUC was calculated by subtracting the blank AUC from the AUC of each sample, the standards, and the positive control. Final ORAC values were expressed as the equivalent concentration of Trolox (TE) that gives the same level of antioxidant activity as the samples at 20 μg/mL. **Means with different alphabets are statistically significant.

and after 24 and 72 hours for any lethality. All procedures were according to the guidelines stated by OECD.

2.10. NF-κB Translocation Assay. NF-kB translocation in MCF-7 cells was examined using NF-kB activation HCS kit which contains Hoechst 33342 and Alexa Fluor 488 conjugated anti-NF-kB dyes. MCF-7 cells were seeded into 96-well plates (Perkin-Elmer Inc., Wellesley, MA, USA) at 6000 cells/well. After overnight, cells were treated with different concentrations of CACF for 1 hr, followed by treatment with 10 μg/mL TNF-α for another 30 minutes. Fixation, permeabilization, and immunofluorescence staining of cells were performed according to the manufacturer's instructions. ArrayScan reader was used to quantify the difference between the intensity of nuclear and cytoplasmic NF-κB-associated fluorescence, reported as translocation parameter.

2.11. Identification and Chemical Analysis Using GC-MS. Gas chromatography mass spectrometry (GC-MS) analysis of the selected fraction was carried out on a Shimadzu GC-17 A network GC system coupled to a mass-selective detector: MS-QP50-50. Separation was conducted on an HP-5 MS column (30 m × 0.32 mm × 3.0 μm), with helium as the carrier gas at a flow rate of 1.0 mL/min. The injection volume was 1 μL with a split ratio of 10 : 1. The column temperature was initially held at 100°C for 3 min and then increased to 290°C at a rate of 10°C/min. The column temperature was then maintained at 290°C for 3 min. The temperatures of the injector and detector were 250°C and 280°C, respectively. Mass acquisition was performed in the range of 40–550 atomic mass units (a. m. u) using electron impact ionization at 70 eV. The major components in this sample were predicted by a spectral database matching against the library of National Institute of Standards and Technology (NIST21 and NIST Wiley).

2.12. Statistical Analysis. Experimental values were expressed as the means ± standard deviation (SD) of the number of experiments indicated in the legends. Statistical significance was assessed using one-way analysis of variance (ANOVA) followed by a multiple comparison test (Tukey's post-hoc test), were $P < 0.001$, $P < 0.01$, and $P < 0.05$ were considered significant. Pearson correlation coefficient was used to assess the correlation between phenolic content and antioxidant activities.

3. Results

3.1. Antioxidant Activity. Table 1 provides a summary of the antioxidant and total phenolic content of CACF. The total phenolic content of CACF was determined to be 37.16 ± 0.85 μg GAE/mg extract.

3.1.1. DPPH Scavenging Activity of CACF. CACF exhibited a significant dose-dependent inhibition of DPPH activity ($P < 0.05$), with an IC_{50} value of 22.56 ± 1.4 μg/mL (Table 1). Maximal DPPH scavenging activity occurred at 41 ± 1.2 μg/mL of CACF with an inhibition of 89%.

3.1.2. Ferric Reducing Antioxidant Power of CACF. CACF showed a significant dose-dependent FRAP value ($P < 0.05$) with a 1048.3 μmol/L for the fraction, while the positive control used in this study displayed a value of 6240 and 907.7 μmol/L for ascorbic acid and BHT, respectively (Table 1).

3.1.3. ORAC Activity of CACF. The area under the curve (AUC) was calculated for oxygen radical absorbance capacity of CACF, trolox, and the positive control quercetin. ORAC results are demonstrated in Table 1. CACF had an ORAC value of 992.34 ± 45.12 μM trolox equivalent at 20 μg/mL. On the other hand, quercetin had an ORAC value of 1018.00 ± 34.82 μM of Trolox equivalent at 5 μg/mL.

3.2. Cytotoxic Activity of CACF. To evaluate the cytotoxic activity, CACF was tested with a series of different doses on nonsmall cell lung cancer (A549), prostate cancer (PC-3), breast cancer (MCF-7), and normal hepatic cells (WRL-68), respectively. After 24 hours, cell viability was determined by the MTT assay. CACF induced cell cytotoxicity in a concentration-dependent manner. These dose titration curves allowed determination of IC_{50} for the CACF towards different cell lines. CACF demonstrated dose-dependent cytotoxic effects with IC_{50} values of 31.42 ± 5.4, 22.61 ± 1.7, 8.1 ± 0.9, and 54.93 ± 8.3 μg/mL; in A549, PC-3, MCF-7 and WRL-68, respectively (Figure 2). In line with these screening, reference drug (Paclitaxel) was used as a positive control whose IC_{50} on tested A549, PC-3, MCF-7 and WRL-68 were 5.675 ± 1.03, 0.37 ± 0.03, 1.583 ± 0.24, and 0.666 ± 0.05, respectively. Hence, these results point out that cell lines vary in their sensitivity.

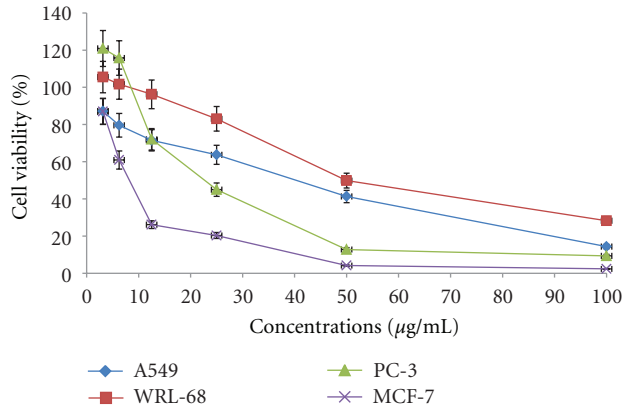

Figure 2: CACF was tested with a series of different doses on A549, MCF-7, PC-3, and WRL-68 cells, respectively. After 24 hours, cell viability was determined by the MTT assay. Test agents induced cell cytotoxicity in a concentration-dependent manner. These dose titration curves allowed determining IC_{50} for the test agents towards different cell lines. The IC_{50} value of CACF on the viability of A549, PC-3, MCF-7, and WRL-68 has been determined to be 31.42 ± 5.4, 22.61 ± 1.7, 8.1 ± 0.9, and 54.93 ± 8.3 μg/mL, respectively.

3.3. In Vitro Inhibitory Effects of CACF on TNF Production and RAW264.7 Cell Viability. Stimulation of RAW264.7 with LPS for 4 hours caused a significant increase in TNF-α production (Figure 3). CACF effectively and dose dependently inhibited TNF-α release with an IC_{50} of 0.012 μg/mL as depicted in Figure 4. CACF exhibited maximal TNF inhibition of 90% at 0.31 μg/mL. This significant inhibitory effect was observed at noncytotoxic doses ranging from 0.031 to 0.002 μg/mL (Figure 5).

3.4. In Vivo Activity

3.4.1. Acute Toxic Effects of CACF. The acute toxicity study revealed the nontoxic nature of CACF. There was a 100% survival rate of the animals treated with CACF at doses ranging from 10 to 1000 mg/kg. Moreover, no toxic effects were observed throughout the study.

3.4.2. The Inhibitory Effect of CACF on Serum TNF. Stimulation of animals with LPS leads to an increase in serum TNF levels up to 2 ng/mL in rats pretreated with PBS alone. On the other hand, animals pretreated with dexamethasone and CACF have shown a significant reduction in TNF levels (Figure 6). Moreover, CACF showed a dose-dependent inhibitory effect of serum TNF in LPS-stimulated rats, and this effect in rats pretreated with 100 mg/kg was almost 61%, to that of dexamethasone 67%, respectively.

3.5. CACF Inhibits NF-kB Activity. In this study, we tested CACF for its *in vitro* inhibitory effects against NF-κB translocation activated by TNF-α and illustrated by HCS assay. CACF displayed significant inhibitory effects on the activation of NF-κB (Figure 7). In parallel, the morphological changes of NF-κB translocation indicated by immunofluorescence staining (Figure 8) showed an inhibitory effect of

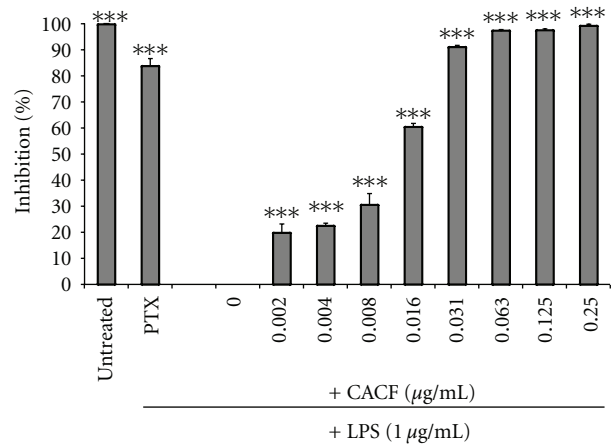

Figure 3: The effects of CACF on TNF production in RAW264.7 cells. Cells were pretreated with the indicated concentrations of CACF, or the TNF inhibitor pentoxifylline (PTX). The cells were stimulated with LPS (1 μg/mL) for four hours or were left untreated (DMSO). The protein concentration was measured using ELISA. Data is representative of three independent experiments and was analyzed using one-way ANOVA with Tukey's post hoc test. The inhibitory effect of CA chloroform fraction was significantly different from stimulated cells (LPS) (***$P < 0.001$).

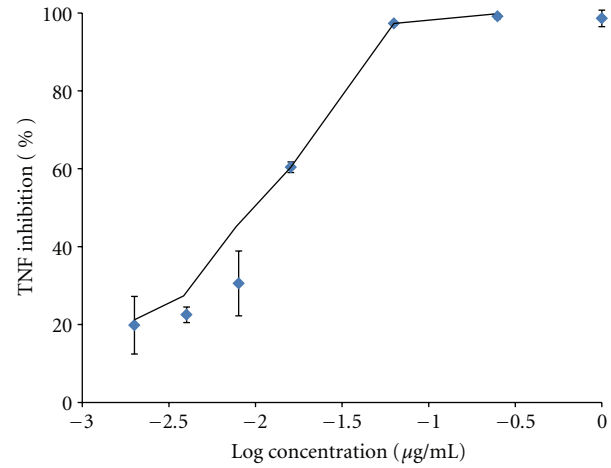

Figure 4: Dose-response effect of the CACF on TNF inhibition: the chloroform fraction had an IC_{50} of 0.012 μg/mL (LOG IC_{50}: −1.931).

CACF on TNF-α-induced NF-κB translocation in a dose-dependent manner. When cells remain untreated, most of the fluorescence staining for NF-κB were in the cytoplasm and rare NF-κB staining found in nuclei area. While cells were stimulated with the TNF-α alone, NF-κB staining significantly increased in nuclei area, suggesting that NF-κB translocated from cytoplasm into the nucleus. However, MCF-7 cells were treated with 8, 4, and 2 μg/mL of CACF, and NF-κB translocation induced by TNF-α was inhibited.

3.6. Chemical Composition. The possible chemical composition of CACF analyzed by GC/MS is presented in (Table 2). Based on the similarity index out of major peaks, total

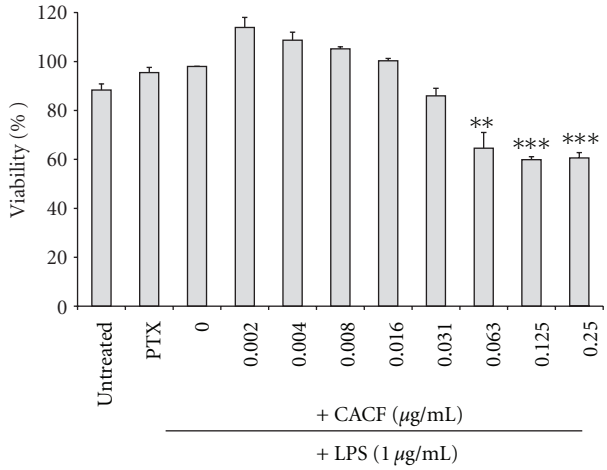

Figure 5: The effects of CACF on RAW264.7 cells' viability. Cells were pretreated with the indicated concentrations of CACF for 4 hours or were left untreated (DMEM). Data is the average of three independent experiments (± SD) and was analyzed using one-way ANOVA with Tukey's posttest (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

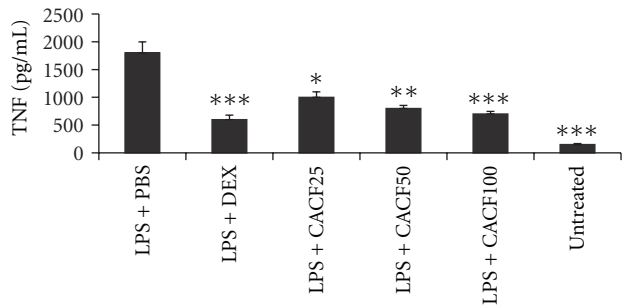

Figure 6: Effect of CACF on serum TNF levels in rats. Different treatment groups were pretreated i.p with 25, 50, and 100 mg/kg of (LPS + CACF), 6 mg/kg of dexamethasone (LPS + DEX) or with phosphate buffer saline (LPS + PBS), and DMSO (Untreated) for 30 mins. All the treatment groups were then either injected with 1 mg/kg of lipopolysaccharides (LPS) or with PBS for 90 mins. Blood was withdrawn, and serum TNF was quantified using ELISA. One-way ANOVA with Tukey's post-analysis was used to calculate the statistical significance among the groups when compared to LPS + PBS. ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$.

22 compounds were detected in this fraction. The most abundant component comprise of 2-Morpholinoethyl isothiocyanate (29.04%), 1,E-11,Z-13-Octadecatriene (16.48%), and Octadecanoic acid, butyl ester (16.15%). Eight compounds (<5%) were not reported in Table 2 due to their minority.

4. Discussion

The current study revealed the pleiotropic bioactivities of the chloroform fraction of *Centratherum anthelminticum* (CACF) seeds. The antioxidant assays performed using the DPPH, ORAC, and FRAP methodologies revealed the free radical-scavenging possibilities of this fraction. Antioxidants

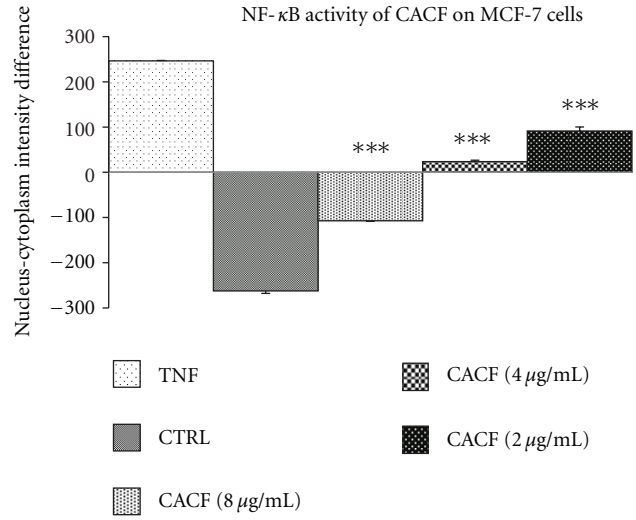

Figure 7: Dose-response histogram of CACF treated MCF-7 cells for 1 hours and then stimulated for 30 minutes with 10 ng/mL TNF-α for quantitative image analysis of intracellular targets.

are substances that may protect cells from the damage caused by unstable molecules known as free radicals. Several factors can lead to the accumulation of free radicals in the cell; examples are certain chemicals, ultraviolet radiations, inflammatory cytokines, and bacterial lipopolysacharides, and other pesticides further generate oxidative stress conditions and extend their contribution in the progression of various ailments including neurodegenerative diseases, atherosclerosis, diabetes, inflammation, and carcinogenesis [19, 25–27]. However, present work demonstrated that CACF fraction is grouped with certain antioxidant compounds. Our results warrant the probability of the CACF as a natural source of antioxidants which could be promising in hunting free radicals and treating diseases related to free radical reactions. Hence, the ability to scavenge free radicals may attenuate the several signalling pathways triggering tissue damage and inflammation, which in turn will have a protective effect on the cells [28, 29].

Having uncovered the antioxidant nature of CACF, we then turned to look at its effects in a living cell system. Cytotoxic screening models provide important preliminary data to select plant extracts or natural compounds with potential anticancer properties [30]. In this study, the cytotoxic effect of CACF was investigated by the addition of the MTT tetrazolium salt [23] to various cancer cell lines previously treated with CACF. CACF showed selective cytotoxic effect on MCF-7 compared with other tested cell lines with low IC_{50} value, nevertheless it was not cytotoxic to normal cell line, WRL-68. These results specify that cell lines differ in their sensitivity to the same CACF, which may be determined by multiple-cell type-specific signalling cascades and transcription factor activities [31].

In addition to that, CACF exhibited inhibition of TNF production both *in vitro* and *in vivo* without affecting cell viability and animal survival, respectively. This also warrants the ethnomedical uses of this plant in the treatment of

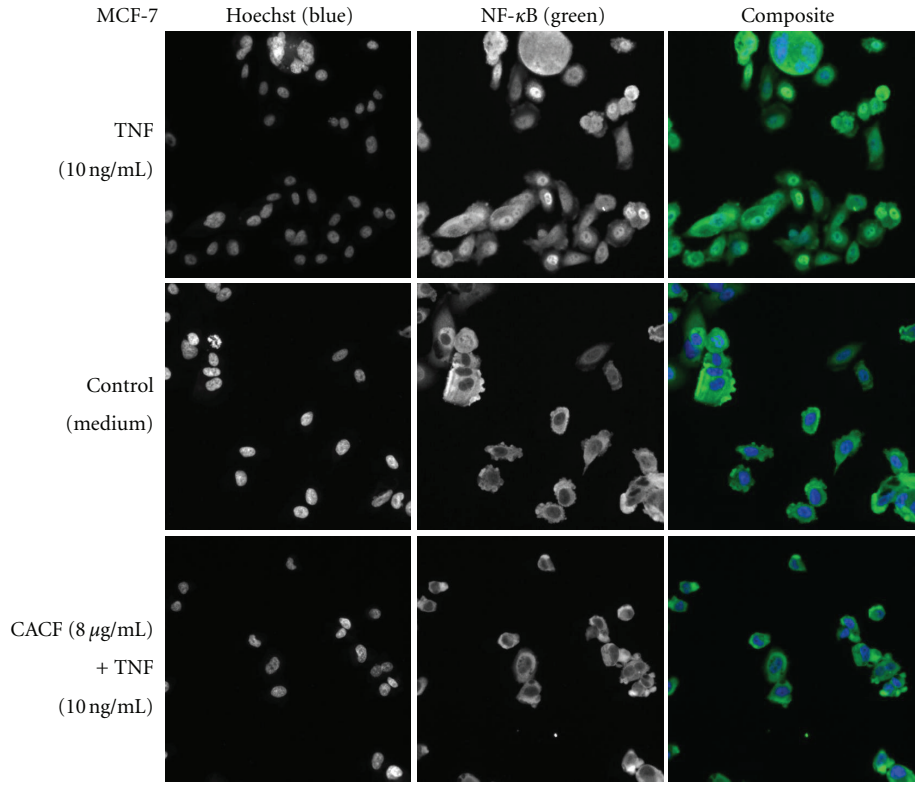

Figure 8: Stained MCF-7 cells were treated with CACF (8 μg/mL) for 1 hour and then stimulated for 30 minutes with 10 ng/mL TNF-α (NF-κB activation).

inflammatory conditions. The ability of CACF to inhibit TNF production may attribute to several factors. Previous studies on *C. anthelminticum* have shown the presence of several bioactive components [18], for example, butein which is one of the secondary metabolites isolated from the seeds of this plant shown to possess both antioxidant and anti-inflammatory activities [14]. The ability of CACF to interfere with inflammatory signalling may in turn explain its cytotoxic effects on cancer cells, since these pathways are also involved in the survival, proliferation, invasion, angiogenesis and metastasis of tumours [32].

Moreover, this fraction along with pleotropic bioactivities demonstrated the *in vitro* inhibitory effects on cell proliferation in MCF-7 cells, which moves our line of study towards carrying out additional experiments to get a better understanding in elucidating mechanisms of breast carcinogenesis with possible future strategies. It is well known that antioxidants are known for their ability to scavenge free radicals from the various stimuli, resulting in activation of transcription factors involved in the transcription of survival and inflammatory genes such as nuclear factor kappa B (NF-κB) [24]. NF-κB is a transcription factor involved in copious inflammatory and cancer-related ailments and has developed as a foremost target in drug discovery [33]. The consequences of NF-κB transcription factors in constitutive activation includes increased survival signalling, cell proliferation, angiogenesis, and invasion, which are key features of the malignant phenotype [34]. Our study demonstrated that CACF in a concentration-dependent manner inhibited constitutive activation of NF-kB translocation stimulated by TNF-α in MCF-7 cells. TNF-α-induced transcript levels for the adhesion molecules, and this might interfer at an early stage of signaling event induced by TNF-α. This suggests that CACF may inhibit the expression of the cell adhesion molecules by interfering with the transcription of their respective genes and may inhibit either the initiation of transcription or the stability of the mRNAs encoding in these molecules. It is well understood that NF-κB is an important transcription factor involved in the gene regulation and contributing in immune and inflammatory responses, including genes encoding [35, 36]. Moreover, tumors with constitutive NF-kB activity have inherent resistance to many anticancer therapies. Thus, NF-κB is believed to play an important role in the regulation of inflammatory response associated with cancer therapy [37, 38].

Analysis of the nonpolar extractable of plant material using GC/MS has been applied before [39]. Our results demonstrate that CACF contains various bioactive components such as 2-morpholinoethyl isothiocyanate which represents 29.04%. Isothiocyanates have been shown to inhibit carcinogenesis and also useful as chemopreventive agents against cancers. They work on a variety of levels. These compounds are shown to induce apoptosis in certain cancer cell lines and in some cases and are even able to induce apoptosis in cells that are resistant to some currently used chemotherapeutic drugs [40–44].

Table 2: Compounds tentatively identified in the chloroform fraction of *Centratherum anthelminticum* (CACF).

Peak number	RT[a]	Percentage of the peak[b]	Molecular weight	Molecular formula	Similarity index	Compound[c]
1	5.279	5.97	116	$C_6H_{12}O_2$	89	2-Pentanone-4-hydroxy-4-methyl
2	8.086	29.04	172	$C_7H_{12}N_2OS$	92	2-Morpholinoethyl isothiocyanate
3	11.278	5.97	180	$C_6H_{12}O_6$	85	d-Allose
4	11.547	3.62	346	$C_{22}H_{34}O_3$	65	Drostanolone AC
5	11.650	1.62	162	$C_{10}H_{10}O_2$	62	3(2H)-Benzofuranone, 2,6-dimethyl
6	15.533	6.36	326	$C_{21}H_{42}O_2$	67	Nonadecanoic acid, ethyl ester
7	17.676	16.48	248	$C_{18}H_{32}$	78	1,E-11,Z-13-Octadecatriene
8	17.933	1.85	312	$C_{20}H_{40}O_2$	68	Hexadecanoic acid, 1,1-dimethylethyl ester
9	17.950	0.45	145	$C_6H_{11}NO_3$	46	Adipic acid monoamide
10	17.967	2.45	282	$C_{12}H_{26}O_5S$	55	d-Mannitol, 1-thiohexyl
11	18.117	0.83	173	$C_7H_{11}NO_2$	45	Ethyl 4-isothiocyanatobutyrate
12	21.450	16.15	340	$C_{22}H_{44}O_2$	85	Octadecanoic acid, butyl ester
13	23.350	1.68	172	$C_9H_{16}O_3$	50	Arabino-hetitol, 2,3 : 5,6-dianhydro-1,7-dideoxy-2,6-di-e-methyl
14	23.942	2.62	298	$C_{17}H_{14}O_5$	42	4H-1-Benzopyran-4-one,5-hydroxy-7-methoxy-2-(3-methoxyphenyl)
Total		95.09				

[a] RT: retention time (min).
[b] Relative area percentage (peak area relative to the total peak area percentage).
[c] Compounds listed in order of their relative area percentage.

In summary, the chloroform fraction of *Centratherum anthelminticum* seed (CACF) displayed a range of interrelated *in vitro* activities, ranging between antioxidant, cytotoxic and *in vitro* and *in vivo* TNF-α inhibition while remaining safe. Furthermore, CACF is maintained concerning mechanistic approach linked with cytotoxic activity on targeted cell line which positively inhibited NF-kB translocation in MCF-7 cells. Therefore, these results merit further pharmacological investigations with detailed phytochemical analysis on *C. anthelminticum*.

Acknowledgments

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CHAPTER 4

CONCLUSION AND FUTURE DIRECTIONS

In search of phytomedicine for diabetes and its associated complications, nine (9) plants were initially screened for their hypoglycemic activity on postprandial hyperglycemia, followed by three *Terminalia* species for potential antioxidant and hypoglycemic effects. Finally, selection of the *Centratherum anthelminticum* seeds among the tested plants with positive effects for the detailed antidiabetic activity, followed by targeted biological activities.

The significant findings in this study which have not been reported elsewhere are:

(1) Amongst the nine (9) plants investigated for hypoglycemic screening, the leaf extracts of three (3) plants - *Centratherum anthelminticum*, *Cissus quadrangularis*, and *Woodfordia fruticosa* Kurz exhibited maximum hypoglycemic activity by reducing postprandial hyperglycemia in normoglycemic rats, without any toxicity. Therefore, it can be suggested that these plants have potential in postprandial hyperglycemia which might help in preventing the development of macro and microvascular complications associated with diabetes.

(2) Study on the leaf extracts of the three (3) selected *Terminalia* species demonstrated antioxidant and hypoglycemic activities, which is probably due to the presence of high polyphenolic contents. This brings new hope in the research on the management of type 2 diabetic complications, and suggest that the use of leaf extracts from these species as food supplements may aid in the reduction of oxidative stress and hyperglycemia.

(3) Based on the pilot screening result of six different plant parts, the defatted crude methanolic fraction of *Centratherum anthelminticum* seeds (CAMFs) was selected for the detailed antidiabetic studies on mouse pancreatic β-TC6 cells, as well as on the diabetic rat models.

(4) Antidiabetic studies on β-TC6 cell lines and *in vivo* studies on type 1 and type 2 diabetic rats demonstrated that CAMFs is helpful in mediating antidiabetic effect by enhancing 2-NBDG glucose uptake, stimulating insulin secretion and inducing higher GLUT-2 and GLUT-4 transporter protein expressions on pancreatic β-TC6 cells. These observations corroborated the beneficial effects of CAMFs by attenuating hyperglycemia and augmenting insulin secretion in the type 2 diabetic rat model. These findings support the use of CAMFs as a possible dietary adjunct for the management of insulin-resistant type 2 diabetes and as a potential source for the discovery of new orally active agent(s) for future diabetes therapy.

(5) Furthermore, to determine the potential of CAMFs on type 2 diabetes and its associated complications, a 12-week study was carried out on type 2 diabetic rats. The study results revealed that CAMFs ameliorated hyperglycemia by down-regulating type 2 diabetic complications such as pro-inflammatory cytokines, oxidative stress and dyslipidemia. In addition, CAMFs inhibited NF-κB translocation in H_2O_2-stimulated β-TC6 cells. Altogether, these findings pave the way for a novel approach to potential treatments of insulin-resistant type 2 diabetes and propose CAMFs as a valuable nutraceutical candidate for type 2 diabetic complications.

(6) Moreover, studies on the chloroform fraction of *Centratherum anthelminticum* seeds (CACF) possesses highest pleotropic bioactivities. Thus, CACF was selected for further studies on TNF-α inhibitory effect and for anticancer potential on RAW264.7 macrophages and certain cancer cells. The results of these studies showed that CACF possesses interrelated activities, ranging between antioxidant, cytotoxic and *in vitro* and *in vivo* TNF-α inhibition effects, without any toxic signs in acute toxicity study. In addition, CACF maintains concerning mechanistic approach linked with cytotoxic activity on MCF-7 cells which positively inhibited NF-κB translocation. Taken together, these findings suggest the use of CACF as an alternative for the management of inflammatory response against elevated TNF-α as well as in oxidative stress conditions. CACF may be helpful in combatting breast cancer, suggestive of its usage after detailed mechanistic studies on targeted MCF-7 cells, including *in vivo* models.

Further studies

This study provides information on different plants that exhibit hypoglycemic and antioxidant potential. However, on the basis of positive results obtained, this study focused on the most potent plant, *Centratherum anthelminticum* seeds for more extensive biological evaluations.

(1) Antidiabetic study on the defatted crude methanolic fraction of *Centratherum anthelminticum* seeds (CAMFs) showed potential antidiabetic properties in the *in vitro* and *in vivo* study models, this activity might attribute to its group of compounds which demonstrate the effects in a synergestic manner. Therefore, it is suggested to isolate different compounds present in CAMFs and evaluate their response on the insulin regulated inflammatory cytokines signaling pathways as well as on the specific targeted proteins through gene expression study. Furthermore, genotoxicity and metabolic

studies with bioavailability test would provide essential safety information on the compounds isolated from the CAMFs.

(2) Pleotropic biological activities on the chloroform fraction of *Centratherum anthelminticum* seeds (CACF) showed potential cytotoxic effects on the human breast cancer cells (MCF-7) through the inhibition of NF-κB translocation. As we know, NF-κB plays an important role in anticancer research and works as a regulator of genes that controls cell proliferation and cell survival. Furthermore, studies on the isolation of active compounds present in CACF is in progress to determine their role on the targeted cancer cells to elucidate their effectiveness on the induction of cell death and apoptosis by utilising different pathways. Moreover, isolated active compounds from CACF would be investigated on *in vivo* mice xenograft model for the anticancer properties.

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Appendix A: Animal ethics approval letter

UNIVERSITI MALAYA
KUALA LUMPUR

يونيورسيتي مالايا

SETIAUSAHA

10 November 2008

Mr Aditya Arya
Jabatan Farmakologi
Fakulti Perubatan
Universiti Malaya

Tuan,

PHYTOPHARMACOLOGICAL EVALUATION OF ISOLATED CONSTITUENTS FROM FEW HERBAL PLANTS EXTRACTS FOR ANTIDIABETIC ACTIVITY IN RELATION TO DIABETES IN NORMAL AND DIABETIC RATS

Dengan sukacitanya Jawatankuasa Etika Penjagaan dan Penggunaan Haiwan, Fakulti Perubatan, Universiti Malaya telah meluluskan permohonan untuk penyelidikan tersebut di atas.

No rujukan etika: **FAR/10/11/2008/AA(R)**

Sila ambil perhatian bahawa nombor rujukan etika yang diberi adalah sah untuk tempoh masa dua tahun sehingga 11 November 2010.

Sila lengkapkan borang yang dilampirkan bersama dengan surat ini (Animal Traffic Record) dan hendaklah dikembalikan kepada pihak kami setelah penyelidikan tamat.

Sekian, terima kasih.

Yang benar,

b/p

Dr. Haji Azizuddin Bin Haji Kamaruddin
Ketua
Pusat Haiwan Makmal
Fakulti Perubatan
Merangkap Setiausaha Jawatankuasa Etika Penjagaan dan Penggunaan Haiwan

SK : **Puan Zura Hazleena Hamizan**
Setiausaha MCRC
Pejabat Dekan
Fakulti Perubatan

Jawatankuasa Penjagaan Dan Penggunaan Haiwan,
Pusat Haiwan Makmal, Fakulti Perubatan, Universiti Malaya,
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UNIVERSITY OF MALAYA
KUALA LUMPUR
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يونيورسيتي ملايا

PEJABAT KETUA

2 Disember 2010

Aditya Arya
Jabatan Farmakologi
Fakulti Perubatan
Universiti Malaya

Tuan,

PERLANJUTAN NOMBOR ETIKA : PHYTOPHARMACOLOGICAL EVALUATION OF ISOLATED CONSTITUENTS FROM FEW HERBAL PLANTS EXTRACTS WITH RELATION TO DIABETES AND NORMAL RAT MODELS

Dengan sukacitanya Jawatankuasa Etika Penjagaan dan Penggunaan Haiwan, Fakulti Perubatan, Universiti Malaya telah meluluskan permohonan untuk perlanjutan nombor etika bagi tujuan penyelidikan tersebut di atas.

No rujukan etika: **FAR/10/11/2008/1210/AA (R)**

Sila ambil perhatian bahawa nombor rujukan etika yang diberi adalah sah untuk tempoh **dua (2) tahun iaitu sehingga 1 Disember 2012.**

Sekian, terima kasih.

Yang benar,

Dr. Haji Azizuddin Bin Haji Kamaruddin
Ketua
Pusat Haiwan Makmal
Fakulti Perubatan
Merangkap Setiausaha Jawatankuasa Etika Penjagaan dan Penggunaan Haiwan

SK : Puan Zura Syazleena Hamizan
Setiausaha MCRC
Pejabat Dekan
Fakulti Perubatan

Appendix B

Declaration forms

1. **Aditya Arya**., Mahmood Ameen Abdullah., Batoul Sadat Haerian., & Mustafa Ali Mohd. (2012). Screening for hypoglycemic activity on the leaf extracts of nine medicinal plants: *in-vivo* evaluation. *E-Journal of Chemistry, 9*(3) 1196-1205.

Authors[a]	Contribution Activities/Percentage	Author Approval Date/Signature[b]
Aditya Arya	All research activities to the project, manuscript preparation 80%	16/10/2012
Mahmood Ameen Abdullah	Animal handling and testing 3%	9/10/2012
Batoul Sadat Haerian	Animal handling 3%	B.S. Haer 10/10/12
Mustafa Ali Mohd	Supervision of overall project 14%	12/10/12

[a]The sequence of the authors is in the accordance of the published paper.

[b]The authors agree that the published article can only be used for Aditya Arya thesis submission.

2. **Aditya Arya**., Shaik Nyamathulla., Mohamed Ibrahim Noordin., & Mustafa Ali Mohd. (2012). Antioxidant and hypoglycemic activities of leaf extracts of three popular *Terminalia* species. *E-Journal of Chemistry, 9*(2) 883-892.

Authors[a]	Contribution Activities/Percentage	Author Approval Date/Signature[b]
Aditya Arya	All research activities to the project, manuscript preparation 80%	16/10/2012
Shaik Nyamathulla	Animal handling 3%	2/10/2012
Mohamed Ibrahim Noordin	Animal testing 3%	10/10/12
Mustafa Ali Mohd	Supervision of overall project 14%	12/10/12

[a]The sequence of the authors is in the accordance of the published paper.

[b]The authors agree that the published article can only be used for Aditya Arya thesis submission.

3. **Aditya Arya.,** Chung Yeng Looi., Shiau Chuen Cheah., Mohd Rais Mustafa., & Mustafa Ali Mohd. (2012). Anti-diabetic effects of *Centratherum anthelminticum* seeds methanolic fraction on pancreatic cells, βTC-6 and its alleviating role in type 2 diabetic rats. *Journal of Ethnopharmacology,* doi:10.1016/j.jep.2012.08.014.

Authors[a]	Contribution Activities/Percentage	Author Approval Date/Signature[b]
Aditya Arya	Overall research activities, manuscript preparation 70%	16/10/2012
Chung Yeng Looi	Bioassay method development 10%	9/10/2012
Shiau Chuen Cheah	Cell viability method development 3%	5/10/2012
Mohd Rais Mustafa	Ideas in the manuscript preparation 7%	9/10/12
Mustafa Ali Mohd	Supervision of overall project 10%	9/10/12

[a]The sequence of the authors is in the accordance of the published paper.

[b]The authors agree that the published article can only be used for Aditya Arya thesis submission.

4. **Aditya Arya.**, Chung Yeng Looi., Shiau Chuen Cheah., Hairin Taha., Mohd Rais Mustafa., & Mustafa Ali Mohd. (2012). The methanolic fraction of *Centratherum anthelminticum* seed downregulates pro-inflammatory cytokines, oxidative stress, and hyperglycemia in STZ-nicotinamide-induced type 2 diabetic rats. *Food and Chemical Toxicology, 50*(11) 4209-4220.

Authors[a]	Contribution Activities/Percentage	Author Approval Date/Signature[b]
Aditya Arya	Overall research activities, manuscript preparation 70%	16/10/2012
Shiau Chuen Cheah	Animal handling 3%	5/10/2012
Chung Yeng Looi	Bioassay method development 3%	9/10/2012
Hairin Taha	Chromatography work 3%	10/10/2012
Mohd Rais Mustafa	Ideas in the research 3%	9/10/2012
Mustafa Ali Mohd	Supervision of overall project 17%	12/10/12

[a]The sequence of the authors is in the accordance of the published paper.

[b]The authors agree that the published article can only be used for Aditya Arya thesis submission.

5. **Aditya Arya**., Mouna Achoui., Shiau Chuen Cheah., Siddig Ibrahim Abdelwahab., Putri Narrima., Syam Mohan., Mohd Rais Mustafa., & Mustafa Ali Mohd. (2012). Chloroform fraction of *Centratherum anthelminticum* (L.) seed inhibits tumor necrosis factor alpha and exhibits pleotropic bioactivities: inhibitory role in human tumor cells. *Evidence-Based Complementary and Alternative Medicine*, doi:10.1155/2012/627256.

Authors[a]	**Contribution Activities/Percentage**	**Author Approval Date/Signature**[b]
Aditya Arya	Overall research activities, manuscript preparation 70%	
Mouna Achoui	Cell viability method development 3%	
Shiau Chuen Cheah	Cytotoxicity assay method development 3%	
Siddig Ibrahim Abdel Wahab	Statistics 3%	
Putri Narrima	Antioxidant assay method development 3%	
Syam Mohan	Animal handling 3%	
Mohd Rais Mustafa	Ideas in the overall research 5%	
Mustafa Ali Mohd	Supervision of overall project 10%	

[a]The sequence of the authors is in the accordance of the published paper.

[b]The authors agree that the published article can only be used for Aditya Arya thesis submission.

Appendix C

Publications

1. Aditya Arya., Mahmood Ameen Abdullah., Batoul Sadat Haerian., & Mustafa Ali Mohd. (2012). Screening for hypoglycemic activity on the leaf extracts of nine medicinal plants: *in-vivo* evaluation. ***E-Journal of Chemistry***, *9*(3) 1196-1205.

2. Aditya Arya., Shaik Nyamathulla., Mohamed Ibrahim Noordin., & Mustafa Ali Mohd. (2012). Antioxidant and hypoglycemic activities of leaf extracts of three popular *Terminalia* species. ***E-Journal of Chemistry***, *9*(2) 883-892.

3. Aditya Arya., Chung Yeng Looi., Shiau Chuen Cheah., Mohd Rais Mustafa., & Mustafa Ali Mohd. (2012). Anti-diabetic effects of *Centratherum anthelminticum* seeds methanolic fraction on pancreatic cells, β-TC6 and its alleviating role in type 2 diabetic rats. ***Journal of Ethnopharmacology***, *144*(1) 22-32.

4. Aditya Arya., Shiau Chuen Cheah., Chung Yeng Looi., Hairin Taha., Mohd Rais Mustafa., & Mustafa Ali Mohd. (2012). The methanolic fraction of *Centratherum anthelminticum* seed downregulates pro-inflammatory cytokines, oxidative stress, and hyperglycemia in STZ-nicotinamide-induced type 2 diabetic rats. ***Food and Chemical Toxicology***, *50*(11) 4209-4220.

5. Aditya Arya., Mouna Achoui., Shiau Chuen Cheah., Siddig Ibrahim Abdelwahab., Putri Narrima., Syam Mohan., Mohd Rais Mustafa., & Mustafa Ali Mohd. (2012). Chloroform fraction of *Centratherum anthelminticum* (L.) seed inhibits tumor necrosis factor alpha and exhibits pleotropic bioactivities: inhibitory role in human tumor cells. ***Evidence-Based Complementary and Alternative Medicine***, doi:10.1155/2012/627256.

6. Aditya Arya., Chung Yeng Looi., Won Fen Wong., Mohamed Ibrahim Noordin., Shaik Nyamathulla., Mohd Rais Mustafa & Mustafa Ali Mohd. (2013). *In vitro* antioxidant, PTP-1B inhibitory effects and *in vivo* hypoglycemic potential of selected medicinal plants. ***International Journal of Pharmacology***, doi:10.3923/ijp.2013.

7. Chung Yeng Looi., **Aditya Arya**., Foo Kit Cheah., Bushra Muharram., Kok Hoong Leong., Khalit Mohamad., Won Fen Wong., Nitika Rai., Mohd Rais Mustafa. (2013). Induction of apoptosis in human breast cancer cells via caspase pathway by vernodalin isolated from *Centratherum anthelminticum* (L.) seeds. **Accepted for publication in PloSONE Journal.**

8. Ismail Adam Arbab., Chung Yeng Looi., Ahmad Bustamam Abdul., Foo Kit Cheah., Won Fen Wong., Mohd Aspollah Sukari., Rasedee Abdullah., Syam Mohan., Suvitha Syam., **Aditya Arya.**, Manal Mohamed Elhassan Taha., Bushra Muharram., Mohd Rais Mustafa and Siddig Ibrahim Abdelwahab. (2012). Dentatin induces apoptosis in prostate cancer cells via Bcl-2, Bcl-xL, survivin downregulation, caspase-9, -3/7 activation, and NF-κB inhibition. ***Evidence-Based Complementary and Alternative Medicine***. doi:10.1155/2012/856029.

Conference

1. Recipient of Graduate Student Travel Award (GSTA) to attend American Society for Pharmacology and Experimental Therapeutics (ASPET) conference held in Anaheim, California, United States of America (USA) in 2010.

2. **Aditya Arya**, Batoul Sadat Haerian & Mustafa Ali Mohamad. (2010). Antihyperglycemic effect of *Centratherum anthelminticum*, *Terminalia chebula* and *Terminalia bellirica* extracts. *The FASEB Journal,* April 2010 24 (Meeting Abstract Supplement) 960.4.

Workshop

1. Attended 15 days workshop on Green Technology (EAGT) held in Taiwan from 1-15 September 2009. Sponsored by Government of China and Department of Science and Technology, Taiwan, 2009.